HUMAN MILK OLIGOSACCHARIDES AS A DEFENSE AGAINST GROUP B *STREPTOCOCCUS*

By

Dorothy LeeAnn Ackerman

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

May 31, 2018

Nashville, Tennessee

Approved:

Steven D. Townsend, Ph.D.

Carmelo J. Rizzo, Ph.D.

Gary A. Sulikowski, Ph.D.

David M. Aronoff, M.D.

In memory of my grandparents,

for exemplifying diligence and perserverence in the face of all hardships

and

To my favorite person, Ed, for being ever steadfast, encouraging, and loving me so well

Life can only be understood backwards; but it must be lived forwards.

Soren Kierkegaard

A man's steps are established by the Lord, and He takes pleasure in his way. Though he falls, he will not be overwhelmed, because the Lord holds his hand.

Psalm 37:23-24

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor Prof. Steven Townsend for taking me as one of his first two graduate students and providing support as I trudged the waters of graduate school. His patience, motivation, and immense knowledge have served to support me in my PhD work. I joined Steve's lab before he officially started his tenure at Vanderbilt, and I will forever be grateful for the risk he took accepting a student he had never met. It is a unique experience to be the first graduate student of a new professor, and I will fondly remember the discussions we had as we worked alongside each other in lab, running columns, and always wishing I was as skilled as him. Steve's consistent encouragement and guidance have pushed me to become the scientist and teacher I am today.

I am also thankful to the other professors who have contributed to my growth and education. I would like to thank my thesis committee, composed of Steve, Carmelo Rizzo, Eva Harth, Gary Sulikowski, and David Aronoff. I am especially thankful to Carmelo Rizzo and his lab for allowing me to use their space and equipment before Steve set up his laboratory. I would like to thank Dr. Aronoff for allowing me to come to their weekly group meetings and absorb as much information about Group B strep that I could. Additionally, I must thank Jennifer Gaddy, who not only provided space and equipment to run biological assays but also served as an excellent female scientist role model. Jen's office was always a safe space to talk about personal and professional struggles and broaden my perspectives. I would be remiss if I did not also thank my undergraduate advisor, Timothy Anstine, who has continued to provide encouragement throughout graduate school.

This work would not have been possible without the financial support of the Vanderbilt

University Department of Chemistry, Vanderbilt Institute of Chemical Biology, and the Mitchum E. Warren Graduate Research Fellowship, along with travel grants from the Amgen Foundation and the Vanderbilt Pre^3 Initiative.

I am grateful to all that I have had the privilege to work with. Ryan Doster, MD, taught me how to run GBS assays, provided a critical eye in data analysis and manuscript preparations, and provided countless stories that made me laugh so hard I sometimes cried. Kelly Craft was gracious enough to let me pass on my biology "hands" to her and helped develop the HMO project. I am grateful to Jenna DeSousa and Schuyler Chambers for the enthusiasm and entertainment they brought my last year and a half. I am also thankful for emotional support Jenna provided and the many "Bachelor nights" we had with Carson. I am deeply indebted to Schuyler for providing wonderful feedback and editing of this document. Jamin Keith has the most intense work ethic and complete knowledge of named reactions and always provided a sounding board for ideas. He also taught me how to swim and did not entirely make fun of my lack of ability. It has been a pleasure to work with other members of the Townsend lab: John Hayes, Caroline Braun, Eric Huseman, and our faithful undergraduate researchers. I must thank Christine Markwalter for being a great friend and model graduate student.

While my support in the scientific community has been of tremendous importance, my family, friends, and church community have proved to be some of the most important sources of encouragement. First, I would like to thank my parents for taking daily calls the first year of graduate school as I adjusted to life in Tennessee. They made a three-day drive (each way) from Montana every summer to visit me in Nashville, and I could not have made it through graduate school without my mom's prayers, letters, packages, and words of encouragement. My house

church at Ethos has given me a weekly escape with fellow graduate students to share my highs and lows and bask in the goodness of Jesus. The Sparks and the rest of my Gateway family took me in and loved me as their own. They have supported me even as the busyness and pressure of graduate school kept me from spending more time with them. Jon and Teresa have become my Tennessee parents and have given me a family and a home away from home. I am thankful to Chad Larson, Kindra Galloway, and Stephanie Simek for commiserating about grad school life with me, providing perspective, encouraging me through failures, and rejoicing with me in my success. Chad has always provided honest insight, even if it wasn't what I wanted to hear. My running and volleyball friends have provided countless memories and much needed escapes from work. All my friends that came to visit me in Nashville when I could not spare the time to travel to them gave me necessary mental escapes, reminded me of the value of relationship, and affirmed who I am at the core. I would also like to thank Ed Butler, my confidant and favorite person. Ed has given me more encouragement that I knew a human being could give. I could not have made it through the last year of graduate school without the friendship, laughter, joy, love, and support he provided. Ed has listened to my rants, made me laugh through tears, and reminded me where my hope comes from.

Grad school is full of stress and uncertainties, but I never knew a day where I was not loved and cared for. I have been reminded daily that I am loved by those around me and by the Lord. Thank you to all who have filled my last five years with joy and memories that I will carry with me forever.

TABLE OF CONTENTS

Page

DEDICATION……ii

ACKNOWLEDGEMENTS……iv

LIST OF TABLES…….....x

LIST OF FIGURES……xii

LIST OF SCHEMES……..xx

LIST OF ABBREVIATIONS……...xxi

CARBOHYDRATE NUMBERING AND NOMENCLATURE…….....xxviii

Chapter Page

I. Group B *Streptococcus*.....1
Introduction.....1
Bacterial Factors that Promote Infection.....5
Methods for Serotyping and Other Classifications.....15
Prevalence.....16
Treatments and Prevention.....18
Summary.....23
References.....25

II. Human Milk Oligosaccharides Categorized by Lewis Blood Groups.....44
Introduction.....44
Human Milk Composition.....44
Nutritional Components.....45
Bioactive Components.....46
Carbohydrate Components.....50
Discovery of HMOs.....52
HMO Structure.....54
Biosynthesis of HMOs.....58
Identification and Characterization of HMOs.....60
Results.....63
Assigning Lewis Blood Groups to Human Milk Samples.....63

Conclusion 66
Methods 66
Purification of HMOs 66
MS and MS/MS Analysis of HMO Samples 67
References 68

Appendix A1: Spectra Relevant to Chapter II 89

III. Human Milk Oligosaccharides Exhibit Antimicrobial and Anti-Biofilm Properties Against Group B *Streptococcus* 96
Introduction 96
Initial Study 100
HMOs Modulate Growth and Viability of S. agalactiae 100
Evaluation of HMO Effect on GBS Biofilm Formation 102
Microscopic Evaluation of Biofilms Grown in the Presence of HMOs .. 103
Conclusions to initial study 108
Expanded Study 110
HMOs Modulate Growth and Viability of S. agalactiae 111
Evaluation of HMO Effect on GBS Biofilm Formation 112
Conclusions to Expanded Study 114
Summary and Conclusions 117
Methods 121
Bacterial Strains and Culture Conditions 121
Bacterial Growth and Viability Analyses 121
Bacterial Biofilm Assays 122
Field-Emission Gun Scanning Electron Microscopy 123
Confocal Laser Scanning Microscopy Analyses 123
References 125

Appendix A2: Figures and Supplemental Data Relevant to Chapter III 133

IV. Synthesis and Biological Evaluation of a Series of Human Milk Glycoconjugates as Antibacterial Agents against Group B *Streptococcus* 142
Introduction 142
Rationale for Synthesis 146
Synthesis of Human Milk Glycoconjugates 149

Synthesis of the Lactose Diol Acceptor 149
Synthesis of Monosaccharide Donors 150
First Round of Glycosylations using Lactose Diol Acceptor 155
Modified Lactose Acceptor 160
Second Round of Glycosylations using Axially Acetylated Lactose Acceptor 160
Deptrotection Strategies for Trisaccharides 162
Evaluation of Biological Activity of Trisaccharides and PEG-Congener 164
Conclusions 179
Experimental Methods 181
Bacterial Strains and Culture Conditions 181
Bacterial Growth and Biofilm Assays 181
Trisaccharide Synthesis 182
References 229

Appendix A3: Figures and Supplemental Data Relevant to Chapter IV 236

V. Conclusions 323
References 328

LIST OF TABLES

Table Page

1. 1 Overview of Early and Late Onset Disease 5

1. 2 GBS Virulence Factors Invovled in Colonization and Infection 7

1. 3 Antibiotics Used to Treat GBS 20

1. 4 Overview of Vaccines in Development or Clinical Trials for GBS 23

2. 1 Comparison of macronutrients in human and bovine milk 45

2. 2 Immune Cells found in Human Milk 49

2. 3 Major Monosaccharide Building Blocks of HMOs 55

2. 4 Lewis blood group determinants. 58

2. 5 Human milk donor designation and assigned Lewis blood groups 65

2. 6 Comparison of assigned Lewis blood group distribution to expected distribution 65

3. 1 Summarization of the effect of HMOs on GBS 109

3. 2 Antimicrobial Activity of HMOs against Three Strains of Group B *Streptococcus* 112

3. 3 Anti-biofilm Activity of HMOs against Three Strains of Group B *Streptococcus* 114

3. 4 Summary of Extent of Growth and Biofilm Inhibition against Three GBS strains 118

4. 1 Fucosylation of Lactose Acceptor 11 156

4. 2 Sialylation of Lactose Acceptor 11 157

4. 3 N-acetylglucosylation of Lactose Acceptor 11 157

4. 4 Galactosylation of Lactose Acceptor 11 158

4. 5 Mannosylation of Lactose Acceptor 11 158

4. 6 PEGylation of Lactose Acceptor 11 159

4. 7 Glycosylations of Lactose Acceptor 44 162

4. 8 Deprotection Strategies for Trisaccharides 163

4. 9 Comparison of ^{1}H NMR spectroscopic data of 3'-fucosyllactose **(1)** 225

4. 10 Comparison of ^{13}C NMR spectroscopic data of 3'-fucosyllactose **(1)** 225

4. 11 Comparison of ^{1}H NMR spectroscopic data of 3'-sialyllactose **(2)** 226

4. 12 Comparison of ^{13}C NMR spectroscopic data of 3'-sialyllactose **(2)** 226

4. 13 Comparison of ^{1}H NMR spectroscopic data of lacto-N-triose **(3)** 227

4. 14 Comparison of ^{1}H NMR spectroscopic data of *epi*-Isoglobotriose **(4)** 228

LIST OF FIGURES

Figure Page

1. 1 Scanning electron micrograph image of GBS. 1

1. 2 Presence of Lancefield Group B Carbohydrate (GBC) in the Cell Wall and Structure of GBC 2

1. 3 Mechanisms of group B *Streptoccocus* cellular adherence and invasion 9

1. 4 Mechanisms of group B *Streptococcus* immune evasion..... 11

1. 5 Structural motif common to all GBS CPS..... 13

1. 6 Capsular Polysaccharide Repeat Units for GBS Serotypes..... 14

1. 7 Global distribution of GBS serotypes in invasive disease in young infants..... 17

1. 8 Distribution of GBS serotypes for A) early onset GBS disease and B) late onset GBS disease 18

1. 9 Structures of Antibiotics Used to Treat Group B Strep 21

2. 1 Comparison of Blood Group Antigens and HMOs..... 53

2. 2 Timeline of HMO research in the 20th century..... 54

2. 3 Structure of Several HMOs 56

2. 4 Biosynthesis of neutral complex human milk oligosaccharides (HMO) 59

2. 5 Comparison of spectra of mothers producing different profiles of HMOs 62

2. 6 Representative MALDI-TOF MS HMO profile and corresponding MALDI-TOF MS/MS of m/z 657 and m/z 1022 for HMOs from an individual donor..... 64

A1. 1 MALDI MS spectra for HMO mixtures isolated from Donors 0, 5, 7, 8, 14, 16, 17, 18, 19, and 20..... 90

A1. 2 MALDI MS spectra for HMO mixtures isolated from Donors 24, 29, 31, 32, 34, 37, 38, 42, and 43 91

A1. 3 MALDI MS/MS spectra of selected *m/z* 657.2 ion for HMO mixtures isolated from Donors 0, 5, 7, 8, 14, 16, 17, 18, 19, and 20 92

A1. 4 MALDI MS/MS spectra of selected m/z 657.2 ion for HMO mixtures isolated from Donors 24, 29, 31, 32, 34, 37, 38, 42, and 43 93

A1. 5 MALDI MS/MS spectra of selected m/z 1022.2 ion for HMO mixtures isolated from Donors 0, 5, 7, 8, 14, 16, 17, 18, 19, and 20 94

A1. 6 MALDI MS/MS spectra of selected m/z 1022.2 ion for HMO mixtures isolated from Donors 24, 29, 31, 32, 34, 37, 38, 42, and 43. 95

3. 1 *S. agalactiae* Capsular Polysaccharide Repeat Units Share Structural Elements of Human Milk Oligosaccharides 99

3. 2 Effect of HMOs isolated from individual milk samples on GBS 10/84 proliferation in Todd Hewitt Broth 101

3. 3 Effect of HMOs isolated from individual milk samples on GBS 10/84 proliferation in Todd Hewitt Broth supplemented with 1% glucose 102

3. 4 HMOs at biologically relevant breast milk concentrations induce changes in biofilm formation of GBS cultures. 104

3. 5 Scanning electron micrographs of biofilm formation after 24 h (250x) 105

3. 6 Scanning electron micrographs of biofilm formation after 24 h (1000x) 106

3. 7 CLSM micrographs comparing biofilm formation of GBS 10/84 107

3. 8 CLSM micrographs comparing apical and base sections of GBS 10/84 biofilms 108

A2. 1 Effect of HMOs isolated from individual milk samples on growth rate/proliferation of GBS 10/84 in Todd Hewitt Broth. 134
A2. 2 Effect of HMOs isolated from individual milk samples on growth rate/proliferation of GBS 10/84 in Todd Hewitt Broth supplemented with 1% glucose 134
A2. 3 HMOs at biologically relevant breast milk concentrations induce changes in biofilm formation of GBS cultures 135
A2. 4 Biomass for *S. agalactiae* strain CNCTC 10/84 after 24 h in THB..... 135
A2. 5 Biomass for S. *agalactiae* strain GB590 after 24 h in THB..... 136
A2. 6 Biomass for S. *agalactiae* strain GB2 after 24 h in THB..... 136
A2. 7 Biomass for S. *agalactiae* strain CNCTC 10/84 after 24 h in THB + 1% glucose..... 137
A2. 8 Biomass for S. *agalactiae* strain GB590 after 24 h in THB + 1% glucose..... 137
A2. 9 Biomass for S. *agalactiae* strain GB2 after 24 h in THB + 1% glucose..... 138
A2. 10 Biofilm to biomass ration for *S. agalactiae* strain CNCTC 10/84 after 24 h in THB..... 138
A2. 11 Biofilm to biomass ratio for S. *agalactiae* strain GB590 after 24 h in THB..... 139
A2. 12 Biofilm to biomass ratio for S. *agalactiae* strain GB2 after 24 h in THB..... 139
A2. 13 Biofilm to biomass ratio for S. *agalactiae* strain CNCTC 10/84 after 24 h in THB + 1% glucose 140
A2. 14 Biofilm to biomass ratio for S. *agalactiae* strain GB590 after 24 h in THB + 1% glucose 140
A2. 15 Biofilm to biomass ratio for S. *agalactiae* strain GB2 after 24 h in THB + 1% glucose 141
4. 1 A general glycosylation mechanism 143
4. 2 Mechanism of 1,2-trans selective glycosylations due to participation of the C2 substitutent 144

4. 3 Leaving groups used in this study..... 145

4. 4 Identification of HMO fractions that inhibit the growth of S. agalactiae strain CNCTC 10/84. 146

4. 5 Human Milk Trisaccharide and Oligosaccharide Synthesis..... 147

4. 6 Milk trisaccharides and congeners prepared in this study..... 148

4. 7 Dimeric structure of stannylene acetals 164

4. 8 Additional Trisaccharides..... 165

4. 9 Antimicrobial assays of *S. agalactiae* strain CNCTC 10/84 at 24 h..... 166

4. 10 Antimicrobial assays of *S. agalactiae* strain GB590 at 24 h 166

4. 11 Antimicrobial assays of S. *agalactiae* strain GB2 at 24 h..... 167

4. 12 Biofilm/biomass ratios for CNCTC 10/84 at 24 h 168

4. 13 Biofilm/biomass ratios for GB590 at 24 he. 168

4. 14 Biofilm/biomass ratios for GB2 at 24 h 169

4. 15 Structures of Additional Carbohydrates Tested against GBS..... 170

4. 16 Antimicrobial assays of *S. agalactiae* strain CNCTC 10/84 at 24 h..... 171

4. 17 Antimicrobial assays of *S. agalactiae* strain GB590 at 24 h 172

4. 18 Antimicrobial assays of *S. agalactiae* strain GB2 at 24 h..... 172

4. 19 Biofilm/biomass ratios for CNCTC 10/84 at 24 h. 173

4. 20 Biofilm/biomass ratios for GB590 at 24 h..... 174

4. 21 Biofilm/biomass ratios for GB2 at 24 h 174

4. 22 Antimicrobial assays of *S. agalactiae* strain CNCTC 10/84 at 24 h..... 175

4. 23 Antimicrobial assays of *S. agalactiae* strain GB590 at 24 h. 176

4. 24 Antimicrobial assays of *S. agalactiae* strain GB2 at 24 h..... 176

4. 25 Biofilm/biomass ratios for CNCTC 10/84 at 24 h 177

4. 26 Biofilm/biomass ratios for GB590 at 24 h 178

4. 27 Biofilm/biomass ratios for CNCTC 10/84 at 24 h 178

A3. 1 Fractionation of HMOs from Donor 42. Fractions were characterized by LCMS. 237

A3. 2 Biofilm to biomass ratio for *Streptococcus* strain CNCTC 10/84 after 24 h..... 238

A3. 3 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 9..... 239

A3. 4 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 9 240

A3. 5 ^{1}H NMR spectra (400 MHz, MeOD) of compound 10 241

A3. 6 ^{13}C NMR spectra (100 MHz, MeOD) of compound 10 242

A3. 7 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 11..... 243

A3. 8 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 11..... 244

A3. 9 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 13..... 245

A3. 10 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 13..... 246

A3. 11 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 14..... 247

A3. 12 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 14..... 248

A3. 13 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 15..... 249

A3. 14 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 15..... 250

A3. 15 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 16..... 251

A3. 16 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 16..... 252

A3. 17 ^{1}H NMR spectra (400 MHz, MeOD) of compound 18 253

A3. 18 ^{13}C NMR spectra (100 MHz, MeOD) of compound 18 254

A3. 19 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 19..... 255

A3. 20 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 19..... 256

A3. 21 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 20.....257

A3. 22 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 20.....258

A3. 23 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 21.....259

A3. 24 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 21.....260

A3. 25 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 23.....261

A3. 26 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 23.....262

A3. 27 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 24.....263

A3. 28 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 24.....264

A3. 29 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 25.....265

A3. 30 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 25.....266

A3. 31 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 27.....267

A3. 32 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 27.....268

A3. 33 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 28.....269

A3. 34 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 28.....270

A3. 35 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 30.....271

A3. 36 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 30.....272

A3. 37 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 31.....273

A3. 38 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 31.....274

A3. 39 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 32.....275

A3. 40 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 32.....276

A3. 41 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 34.....277

A3. 42 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 34.....278

A3. 43 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 36.....279

A3. 44 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 36.....280

A3. 45 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 37.....281

A3. 46 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 37.....282

A3. 47 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 38.....283

A3. 48 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 38.....284

A3. 49 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 39.....285

A3. 50 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 39.....286

A3. 51 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 40.....287

A3. 52 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 40.....288

A3. 53 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 41.....289

A3. 54 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 41.....290

A3. 55 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 42.....291

A3. 56 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 42.....292

A3. 57 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 43.....293

A3. 58 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 43.....294

A3. 59 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 44.....295

A3. 60 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 44.....296

A3. 61 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 45.....297

A3. 62 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 45.....298

A3. 63 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 46.....299

A3. 64 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 46.....300

A3. 65 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 47.....301

A3. 66 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 47.....302

A3. 67 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 48 303

A3. 68 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 48 304

A3. 69 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 49 305

A3. 70 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 49 306

A3. 71 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 50 307

A3. 72 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 50 308

A3. 73 ^{1}H NMR spectra (400 MHz, H_2O) of compound 1 309

A3. 74 ^{13}C NMR spectra (100 MHz, H_2O) of compound 1 310

A3. 75 ^{1}H NMR spectra (400 MHz, H_2O) of compound 2 311

A3. 76 ^{13}C NMR spectra (100 MHz, H_2O) of compound 2 312

A3. 77 ^{1}H NMR spectra (400 MHz, H_2O) of compound 3 313

A3. 78 ^{13}C NMR spectra (100 MHz, H_2O) of compound 3 314

A3. 79 ^{1}H NMR spectra (400 MHz, H_2O) of compound 4 315

A3. 80 ^{13}C NMR spectra (100 MHz, H_2O) of compound 4 316

A3. 81 ^{1}H NMR spectra (400 MHz, H_2O) of compound 5 317

A3. 82 ^{13}C NMR spectra (100 MHz, H_2O) of compound 5 318

A3. 83 ^{1}H NMR spectra (400 MHz, H_2O) of compound 6 319

A3. 84 ^{13}C NMR spectra (100 MHz, H_2O) of compound 6 320

A3. 85 ^{1}H NMR spectra (400 MHz, H_2O) of compound 7 321

A3. 86 ^{13}C NMR spectra (100 MHz, H_2O) of compound 7 322

LIST OF SCHEMES

Scheme Page

4. 1 Synthetic approach to human milk trisaccharides functionalized at the 3’ position of lactose 149

4. 2 Synthesis of Lactose Diol Acceptor 150

4. 3 Synthesis of Fucosyl Fluoride Donor 152

4. 4 Synthesis of Sialyl Phosphite Donor 152

4. 5 Synthesis of Sialyl Thiol Donor 153

4. 6 Synthesis of Glucosamine Imidate Donors 153

4. 7 Synthesis of Galactose and Glucose Imidate Donors 154

4. 8 Synthesis of Thioethyl Galactose Donor 154

4. 9 Synthesis of Thiophenyl Mannose Donor 154

4. 10 Synthesis of Tosylated PEG Donor 155

4. 11 Synthesis of Axially Acetylated Lactose Acceptor 160

LIST OF ABBREVIATIONS

1-4GalT1	β1-4 galactosyltransferase
2'-FL	2'-fucosyllactose
3-FL	3-fucosyllactose
3'-SL	3'-sialyllactose
6'-SL	6'-sialyllactose
AAP	American Academy of Pediatrics
Ac	acetyl
ACOG	American College of Obstetricians and Gynecologists
ACP	alpha C protein
$AgBF_4$	silver tetrafluoroborate
$AgClO_4$	silver perchlorate
$AgCO_3$	silver carbonate
AgOTf	silver trifluoromethanesulfonate
Alp	alpha-like protein
AMP	antimicrobial peptide
ANOVA	analysis of variance
Aq.	aqueous
$BF_3 \bullet Et_2O$	boron trifluoride diethyl etherate
BibA	GBS immunogenic bacterial adhesin
Bn	benzyl
BnBr	benzyl bromide
Bu_4NI	tetrabutylammonium iodide
°C	degrees Celsius
C4bp	C4-binding protein
CAMP	Christie Atkins Munch Peterson factor
CCL4	C-C motif ligand 4
CD14	cluster of differentiation 14
CDC	Center for Disease Control and Prevention

$CDCl_3$	chloroform-*d*
CIP	contact ion-pair
CLSM	confocal laser scanning microscopy
CNCTC	Czech National Collection of Type Cultures
CovR/S	cluster of virulence responder/sensor
Cp_2HfCl_2	bis(cyclopentadienyl)halfnium(IV) dichloride
CPS	capsular polysaccharide
CspA	cell-surface protease A
CV	crystal violet
CXCL1	C-X-C motif ligand 1
CZE	capillary zone electrophoresis
d	doublet
DAST	diethylaminosulfur trifluoride
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dd	doublet of doublets
ddd	doublet of doublet of doublets
DF-L	difucosyllactose
DMF	dimethylformamide
DMTST	dimethyl(methylthio)sulfonium trifluoromethanesulfonate
DNA	deoxyribonucleic acid
DSLNT	disialolacto-N-tetraose
dt	doublet of triplets
ECM	extracellular matrix
EGF	epidermal growth factor
EOD	early onset disease
Epo	Erythropoietin
eq.	equivalent
ESI-TOF	electrospray ionization time-of-flight

EtOAc	ethylacetate
EtOH	ethanol
EtSH	ethane thiol
FOS	fructo-oligosaccharide
FsbA/B	fibrinogen-bidning proteins A and B
Fuc	fucose
FucT	fucosyltransferase
FUT2	α-1,2 fucosyltransferase
FUT3	α-1,3/4 fucosyltransferase
G-CSF	granulocyte-colony stimulating factor
g	gram
Gal	galactose
GBC	Group B carbohydrate
GBS	Group B *Streptococcus*, *Streptococcus agalactiae*
GC	gas chromatography
GD3	ganglioside precursor disialohematoside
Glc	glucose
GlcNAc	N-acetyl glucosamine
GM1/3	monosialotetrahexosylganglioside
GM3	monosialodihexosylganglioside
GOS	galactooligosaccharide
Groα	growth-related oncogene-α
GSK	GlaxoSmithKline
h	hour
HBr	hydrobromic acid
HCl	hydrochloric acid
$Hg(OAc)_2$	mercury (II) acetate
HILIC	hydrophilic interaction chromatography

HIV	human immunodeficiency virus
HMO	human milk oligosaccharide
HPAEC	high-pH anion-exchange chromatography
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IAP	intrapartum antibiotic prophylaxis
IC50	half-maximal inhibitory concentration
ICE	integrative and conjugative element
IgG/M	immunoglobulin G and M
IL	interleukin
INFγ	interferon γ
J	coupling constant
L	liter(s)
LCMS	liquid chromatography-mass spectrometry
Le	Lewis
LG	leaving group
Lmb	laminin-binding protein
LNDF-I	lacto-N-difucohexaose I
LNF-I, II, III, V	lacto-N-fucopentaose I, II, III, V
LNH	lacto-N-hexaose
LNnT	lacto-N-neotetraose
LNT	lacto-N-tetraose
LOD	late onset disease
LRMS	low resolution mass spectrometry
LSTa, b, c, d	sialyl-lacto-N-tetraose a-c
LTA	lipoteichoic acid
m	milli, multiplet (NMR)
M	moles per liter

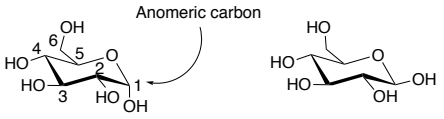
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight mass spectrometry
MeCN	acetonitrile
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MGE	mobile genetic element
$MgSO_4$	magnesium sulfate
MHz	megahertz
min	minutes(s)
MIP1β	macrophage inflammatory protein β
MLST	multilocus sequence typing
mol	mole(s)
mp	melting point
MS/MS	tandem mass spectrometry
MUC1/4	mucin 1 and 4
NaH	sodium hydride
NAM	N-acetyl muramic
NBS	N-bromosuccinimide
NEC	necrotizing entercolitis
Neu5Ac	N-acetylneuraminic acid
NIS	N-iodosuccinimide
NMR	nuclear magnetic resonance
NPC	normal-phase chromatography
OD560	optical density at 560 nm
OD600	optical density at 600 nm
PCR	polymerase chain reaction
PEG	polyethylene glycol
PG	protecting group

PGC	porous graphite carbon chromatography
pH	potential of hydrogen
PilA	pilus-associated adhesin
ppm	parts per million
q	quartet
RANTES	regulated on activation, normal T cell expressed and secreted
RCOG	Royal College of Obstetricians and Gynecologists
RNA	ribonucleic acid
ROS	reactive oxygen species
ROUT	robust regression and outlier removal
RPC	reversed-phase chromatography
s	singlet
ScpB	C5a peptidase
Se	secretor
SEM	scanning electron microscopy
SEM	standard error of the mean
Sia	sialic acid
sIgA	secretory immunoglobulin A
Siglec	sialic acid-binding immunoglobulin-type lectins
$SnCl_2$	tin (II) chloride
$SnCl_4$	tin (IV) chloride
SodA	superoxide dismutase
Srr	serine-rich repeat domain protein
SSIP	solvent-separated ion-pair
Stk1	serine/threonine kinase
TCS	two component system
TfOH	trifluoromethanesulfonic acid
TGF-β2	transforming growth factor

THB	Todd Hewitt Broth
TLC	thin layer chromatography
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TNF-α	tumor necrosis factor α
Troc	2,2,2-trichloroethoxycarbonyl
Ts	4-toluenesulfonyl
UDP-Gal	uridine diphosphate galactose
UPEC	uropathogenic Escherichia coli
VEGF	vascular endotheial growth factor
β-H/C	β-hemolysin/cytolysin
δ	chemical shift in ppm

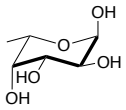
CARBOHYDRATE NUMBERING AND NOMENCLATURE

Monosaccharides can exist as two isomers, due to mutarotation about the anomeric carbon. The numbering of monosaccharides is shown below. They are termed α when the substituent at C1 and C5 are trans and β when the substituent at C1 and C5 are cis. N-acetylneuraminic acid is unique because C1 is assigned to the carbon of the carboxylic acid, and the anomeric carbon is C2.

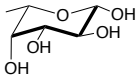


α-D-glucopyranose

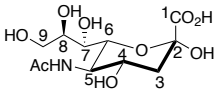
β-D-glucopyranose



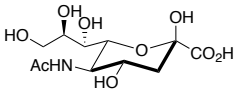
α-L-fucopyranose



β-D-fucopyranose

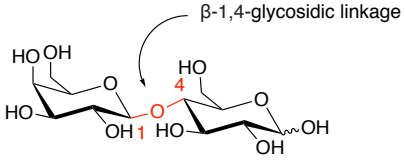


N-Acetyl-α-Neuraminic acid

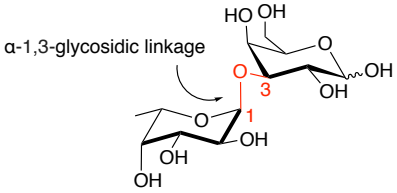


N-Acetyl-β-Neuraminic acid

When two monosaccharides are joined, they form a glycosidic linkage between an alcohol of one residue and the anomeric center of another. A glycosydic linkage is labeled by the carbons to which they are attached. For example, in lactose, the galactose is linked to glucose in a β-1,4 linkage. This can be represented in shorthand by Galβ1-4Glc, indicating that the anomeric carbon (C1) of galactose is linked to C4 of glucose in a β linkage. The α and β designation is determined the same way as monosaccharides.



Lactose (Gal-β-1,4-Glc)



3-Fucosylgalactose (Fuc-α-1,3-Glc)

CHAPTER I

Group B *Streptococcus*

Introduction

Streptococcus agalactiae, more commonly known as Group B *Streptococcus* (Group B Strep, GBS), is a Gram-positive pathogen that divides along a single axis to cause growth in a chain-like fashion. (Figure 1.1).[1] According to the Lancefield classification system, the presence of the group B carbohydrate (GBC) antigen in the cell wall identifies *Streptococcus agalactiae* as a Group B *streptococococcus* (Figure 1.2).[2]

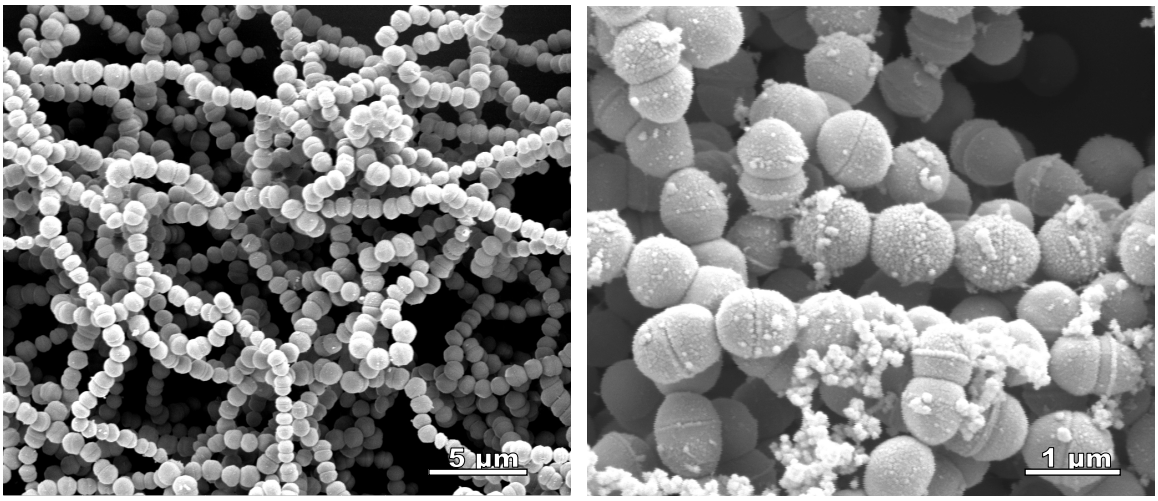


Figure 1. 1 Scanning electron micrograph image of GBS. Chain-like formations and division along a single axis are shown at 2,000x and 10,000x magnification (Image courtesy of Dr. Ryan Doster)

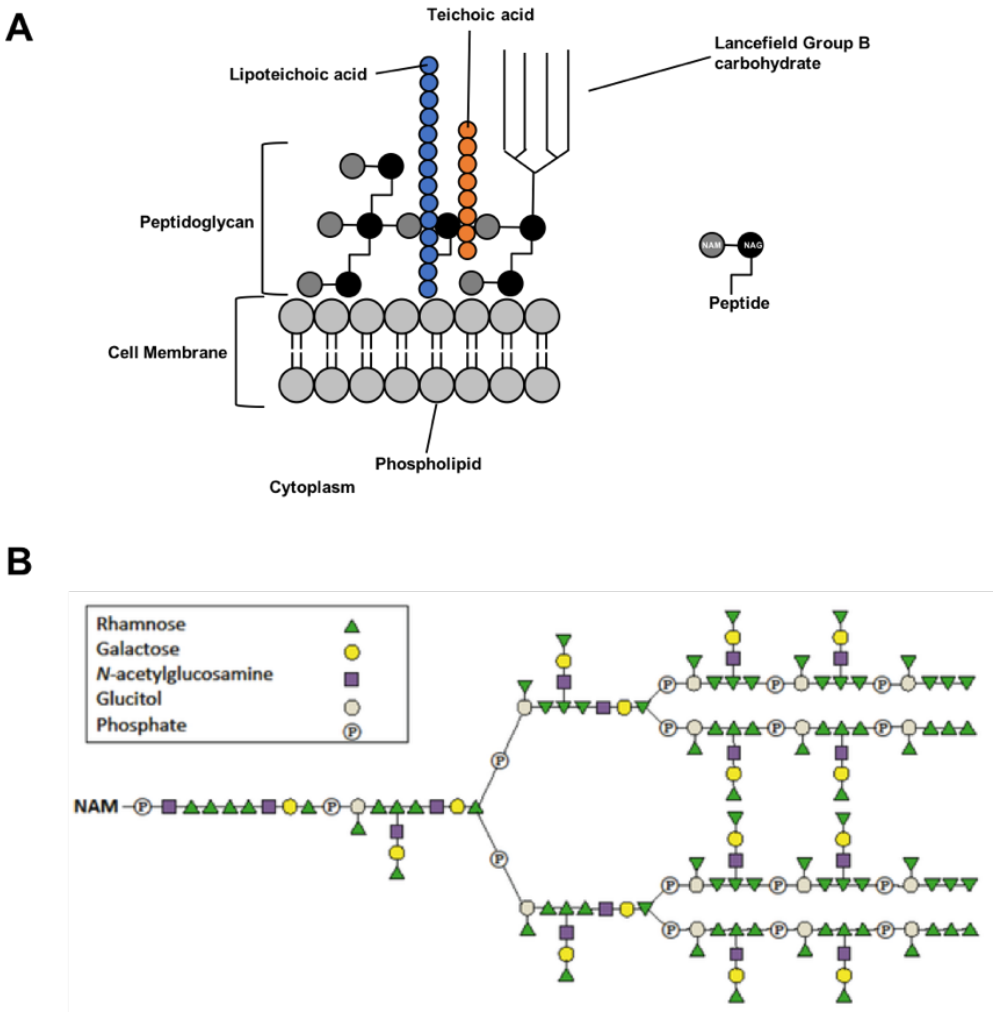


Figure 1. 2 Presence of Lancefield Group B Carbohydrate (GBC) in the Cell Wall and Structure of GBC. A) GBS is attached to the peptidoglycan of the cell wall via linkage to the N-acetyl muramic (NAM) moiety, a component of the peptidoglycan. B) The structure of the multiantennary GBC is shown linked to NAM. (Adapted from Caliot, E., Dramsi, S., Chapot-Chartier, M-P, Courtin P, Kulakauskas S, et al. (2012) Role of the Group B Antigen of *Streptococcus agalactiae*: A Peptidoglycan-Anchored Polysaccharide Involved in Cell Wall Biogenesis. PLoS Pathog 8(6): e1002756. doi:10.1371/journal.ppat.1002756.)

Group B Strep is typically associated with infection during the perinatal period and is a leading cause of neonatal infections.[1, 3, 4] Before emerging as a prominent human pathogen, GBS was first identified as a cause of bovine mastitis in 1887.[5] Mastitis in an infection of the mammary glands and can lead to a decrease in the quantity and quality of milk produced.[5] Bovine GBS

infections usually respond to treatments of penicillin-type antibiotics with cure rates ranging from 75-95%, allowing for efficient control of bovine mastitis outbreaks.[6-8]

It was not until 1935, almost 50 years after the first reported case of bovine infection, that human infection in the vaginal tracts of pregnant women was reported.[1, 9] Soon after, GBS infection emerged as a cause of fatality in post-partum women.[10] Still, few cases of infant-related diseases were reported until the early 1970s when GBS became the leading cause of neonatal sepsis and meningitis in the United States.[10, 11] During this decade, the infant mortality rate due to GBS-related diseases reached a high of 55%.[11] Over the next few decades, strategies and guidelines for preventing, diagnosing, and treating GBS were developed by identifying and analyzing risk factors for both mother and neonate and using intrapartum antibiotic prophylaxis to prevent transmission. Intrapartum antibiotic prophylaxis is the administration of antibiotics during the labor and delivery period. This method of prevention is used to reduce the bacterial burden of maternal colonization, which in turn reduces the possibility of vertical transmission from mother to infant.[12]

Several risk factors have been associated with maternal and neonatal GBS infections as well as transmission. Age, race, gender, pregnancy outcomes, and previous incidences of colonization or infection contribute to development of GBS disease.[10, 11, 13] While few risk factors are prevalent enough to be used in prevention strategies, awareness of the conditions associated with GBS infections has proved insightful for understanding the perinatal pathophysiology of GBS.[10] For instance, maternal colonization is a significant determination of vertical transmission and infection in the infant.[13] This connection has led to administration of antibiotics to colonized mothers to prevent transmission.

GBS colonizes between 10-40% of adults, but it is commonly asymptomatic in healthy

individuals.[10, 14, 15] However, it can cause severe infections in immunocompromised individuals, especially infants who have an immature immune system. GBS colonizes the gastrointestinal and genitourinary tracts of adults but colonizes the gastrointestinal tract and throats of infants.[13] Colonization of genitourinary tracks does not occur until after puberty, and neonatal infections in throats arise after fetal aspiration or ingestion of GBS infected amniotic fluid and contraction during descent through the birth canal.[16]

Infections caused by GBS during pregnancy can lead to several adverse outcomes including stillbirth, chorioamnionitis, and preterm birth.[1] Choriamnionitis, an inflammation of fetal membranes due to a bacterial infection, can result from an ascending infection of maternal vaginal GBS colonization. Intrauterine infections can induce premature labor.

Neonatal diseases caused by GBS occur either as early onset disease (EOD) or late onset disease (LOD), depending on the age at which an infant acquires the infection. While EOD typically presents within the first 24 hours after birth and can occur up to seven days old, LOD presents after the first 7 days of life.[13] A 2017 meta-analysis reported global incidence risks to be 0.41 for EOD and 0.26 for LOD per 1000 live births.[17] EOD is associated with vertical transmission that originates from maternal colonization. Methods of vertical transmission include aspiration of contaminated fluids during birth or *in utero* via bacterial ascension into the amniotic sac through ruptured membranes or translocation through intact membranes.[10, 11] EOD manifests mainly as sepsis and pneumonia, but can develop into meningitis.[18-20] LOD can manifest in a similar manner to EOD, but predominantly occurs as meningitis.[21] Unlike EOD, the transmission of LOD is less understood but is also associated with maternal colonization and prematurity.[18, 22-24] LOD is not always acquired vertically from the mother; instead, it can also be acquired through

horizontal transmission from maternal, hospital, and community sources.[20]

Table 1. 1 Overview of Early and Late Onset Disease

	Incidence Risk (per 100 live births)	Infant Age	Manifestations
EOD	0.41	0 – 7 days	Sepsis Pneumonia
LOD	0.26	> 7 days	Meningitis

Bacterial Factors that Promote Infection

Genome sequencing of GBS has revealed the presence of at least 21 two-component systems (TCS).[25-27] TCS are the most common signal transduction systems found in bacteria and are composed of a membrane-bound histidine kinase and a response regulator.[18] For signal transduction to occur, the membrane-bound histidine kinase first recognizes an external stimulus and responds by phosphorylating the corresponding response regulator. The phosphorylation of this response regulator leads to a change in gene expression and subsequent adaptation to the bacteria's environment.[18] Signal transduction systems are responsible for regulating gene expression and other cellular responses. Roles of only five of these two-component systems (CovR/CovS, RgfC/FgrA, DltR/DltS, CiaR/CiaH, and FspR/FspS) have been elucidated.[18, 27]

The CovR/CovS TCS regulates several virulence factors including production of pore-forming toxins, escape of host recognition by expression of capsular polysaccharides (CPS) and C5a peptidase (ScpB), evasion of host-produced radical oxygen species (ROS), and increased binding to host cells with fibrinogen-binding protein A (FbsA).[18, 28-32] The RgfC/FgrA TCS also

plays a role in the expression and regulation of FbsA.[25, 27, 33, 34] Resistance to antimicrobial peptides (AMPs) through alanylation of lipotechoic acid is regulated by the DltR/DltS TCS.[35, 36] The specific roles of CiaR/CiaH are less understood, but this TCS is known to aid in GBS intracellular survival and evasion of environmental stresses such as ROS and AMP.[37, 38] FspR/FspS, a recently identified TCS, is necessary for vaginal persistence of the bacteria through regulation of genes involved in sugar transport.[27] Many virulence factors of GBS are regulated by one or several of these two-component systems.

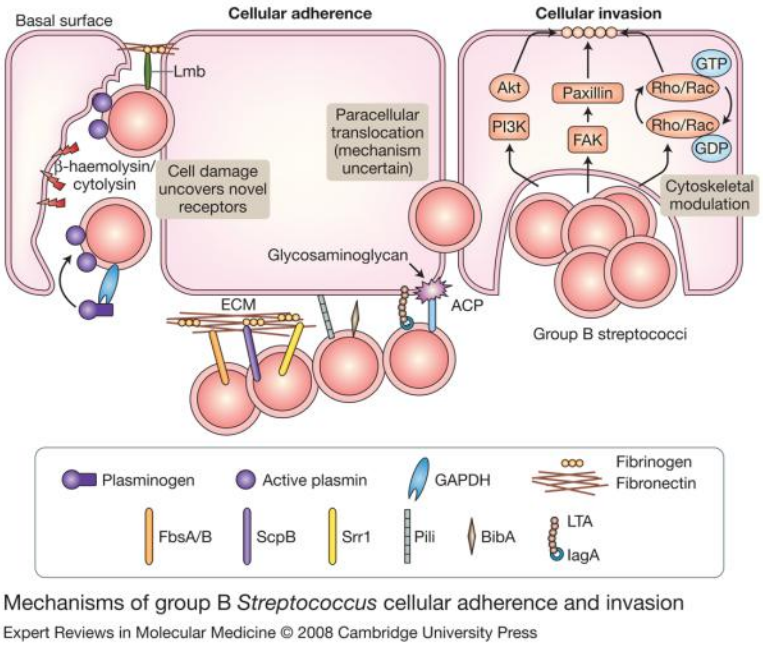
The genome of GBS contains over 100 signaling systems and transcriptional regulators, which position GBS to colonize and survive in multiple environments.[25, 34] In addition to the presence of genes encoding TCS, GBS encodes at least six other independent regulators of gene expression.[18] These other systems are regulated by activities such as changes in concentration of biomolecules in the cytosol or fluctuation of other environmental factors.[34, 39, 40] Increased studies of these systems will improve the understanding of GBS colonization and infection and provide targets for therapeutics to combat colonization and subsequent disease states.

GBS has several importance virulence factors that contribute to its pathogenesis, which can be divided into three stages: 1) adherence to epithelial surfaces, 2) invasion and host damage, and 3) survival *in vivo*. An overview of these virulence factors is presented in Table 1.2.

Table 1. 2 GBS Virulence Factors Invovled in Colonization and Infection

Virulence Factor	Mode of Action	Host Target
Fibrinogen-binding proteins	Promote adherence (FbsA, FbsB, FbsC) Promote entry into host cells (FbsB)	Fibrinogen
Laminin-binding proteins	Promote adherence (Lmb) Promote entry into host cells (Lmb)	Laminin
Serine rich repeat protein	Promote adherence to epithelial cells (Srr-1) Enhance virulence (Srr-2)	Fibrinogen
Immunogenic bacterial adhesion (BibA)	Promote adherence of GBS Binds complement regulatory protein C4bp	C4-binding protein
Pili	Promote resistance to AMP Promote adherence to hose cells	Collagen I
Capsular polysaccharide	Prevent recognition of GBS through molecular mimicry of host-cell surface glycoconjugates Masks pro-inflammatory cell wall components	Siglecs
α-C protein	Facilitates GBS adherence to host epithelial cells	Host cell surface glycosaminoglycan
Pore-forming toxins β-H/C, CylE, CfB (CAMP factor)	Promotes invasion of host cells and triggers host-cell lysis Impair cardiac and liver function Induces inflammatory responses and apoptosis Forms pores in host-cell membrane (CAMP) Binds to GPI anchored proteins (CAMP)	Cell membranes Host phagocytes CAMP
Serine protease (CspA)	Cleaves fibrinogen and chemokines Impairs neutrophil recruitment and phagocytic killing of GBS	Fibrinogen
Peptidase (C5a) (ScpB)	Prevents neutrophil recruitment due to cleavage of complement C5a Promotes adherence by binding to ECM fibronectin and epithelial cells	Complement component C5a Fibrinonectin
Superoxide dismutase (SodA)	Detoxifies singlet oxygen and superoxide	ROS generated by phagocytes

While GBS can adhere to a variety of human cells, the acidic pH of the vaginal mucosa is optimal for enhanced GBS attachment.[26, 41] The CovR/CovS system responds to changes in pH and increases regulation of virulence factors in acidic environments.[1] Adherence of GBS is also mediated by interactions between GBS surface proteins and host extracellular matrix (ECM) components, which allows subsequent invasion of the host cell (Figure 1.3).[18] Similar to other related Gram-positive pathogens, both fibrinogen- and laminin-binding proteins are known to mediate adherence of GBS to the host.[26] GBS binds to fibrinogen, a host glycoprotein produced in response to inflammation, by several fibrinogen-binding proteins (FbsA, FbsB, and FbsC).[1, 18, 42] Laminin-binding protein (Lmb) facilitates GBS binding to host-cell laminin, allowing for GBS attachment to the host basal membrane.[18, 43] Adherence of GBS to host cells is also mediated by serine-rich repeat proteins (Srr), a family of glycoproteins that bind to host epithelial cells through interactions with host fibrinogen.[1, 18, 44-46] GBS has four different forms of cell-surface immunogenic bacterial adhesins (BibA) that facilitate adherence of GBS to host cells.[18, 47] BibA binds to C4-binding protein (C4-bp), a regulator of the classical complement pathway.[18, 47] Another adhesin, PilA, is a GBS pili that mediates adherence by binding to the host cell.[1, 18, 48] Pili are long appendages that protrude outside the capsule and cover the bacterial surface. Host sialic-acid-recognizing immunoglobulin super family lectins (Siglecs) are also able to recognize and bind the sialic acid rich capsule of GBS.[1, 49] Adherence and colonization of the vaginal mucosa furthers the risk of vertical transmission from mother to infant.



Mechanisms of group B *Streptococcus* cellular adherence and invasion

Figure 1. 3 Mechanisms of group B *Streptococcus* cellular adherence and invasion. Surface-expressed proteins FbsA/B, ScpB, Srr1, pili, BibA, LTA and ACP mediate group B *Streptococcus* (GBS) binding to host cells and ECM components, such as fibrinogen and fibronectin. Secreted β-haemolysin/cytolysin promotes GBS invasion, possibly by breaking down host barriers to reveal novel receptors on the basement membrane, such as laminin. GBS also use GAPDH to activate host plasminogen and degrade the ECM. Intracellular GBS invasion is enhanced by bacterial-dependent cytoskeletal rearrangements triggered by host PI3K/AKT- and FAK-signalling pathways and the Rho family of GTPases. Alternatively, GBS can also use an unknown mechanism to cross host epithelial barrier by a paracellular route. Several GBS adhesins, including FbsB, ScpB, pili, LTA and ACP, also contribute to cellular invasion. Abbreviations: ACP, alpha C protein; BibA, GBS immunogenic bacterial adhesin; ECM, extracellular matrix; FAK, focal adhesion kinase; FbsA/B, fibrinogen-binding proteins A and B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Lmb, laminin-binding protein, LTA, lipoteichoic acid; PI3K, phosphoinositide 3-kinase; ScpB, C5a peptidase; Srr1, serine-rich repeat domain protein 1. (Reproduced with permission from Maisey HC, Doran KS, Nizet V. Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. *Expert Reviews in Molecular Medicine*. 2008;10:e27. doi:10.1017/S1462399408000811.)

After adherence to host cells, GBS invasion and host damage can result from bacterial excretion of toxins and invasins (Figure 1.3). In addition to playing a role in GBS adhesion, FbsB and Lmb have been shown to promote GBS entry into epithelial cells.[18, 49-51] Another protein that mediates GBS invasion of epithelial cells is alpha C protein (ACP), a surface-anchored protein that binds to host cell glycosaminoglycans.[1, 18, 49, 52-54] Invasion of cells after damage results from

the action of pore-forming toxins. Two important pore-forming toxins of GBS are β-hemolysin/cytolysin (β-H/C) and Christie Atkins Munch Peterson (CAMP) factor.[18] β-H/C lyses epithelial and endothelial cells causing direct tissue injury and promoting intracellular invasion.[18, 26, 49] CAMP factor oligomerizes in the target membrane to form pores and provoke cell lysis.[18, 26] Interestingly, while CAMP factor aids in cell lysis, it has been shown to be unnecessary for systemic virulence of GBS, suggesting that CAMP factor is only essential when β-H/C activity is attenuated.[18, 55] In addition to invasion through host damage, GBS has also been shown to participate in paracellular transport across membranes via transient modification of junctional complexes in the monolayer that are able to reassociate after bacterial translocation.[49, 56]

In order for GBS to survive *in vivo*, bacteria must evade the host immune system while also maintaining access to nutrients necessary for survival and proliferation (Figure 1.4). Several of the virulence factors involved in GBS adherence and invasion contribute to bacterial survival *in vivo*. For example, GBS pili aid in adherence to host cells and play a role in mediating GBS resistance to AMPs.[49, 57] Additionally, GBS evade AMPs by engaging the *Dlt* operon which leads to incorporation of excess D-alanine into the lipotechoic acid (LTA) component of the cell wall, decreasing the net negative charge on the surface of the bacteria (Figure 1.4).[49] The innate immune system recruits phagocytes such as neutrophils and macrophages to the site of infection to clear bacterial pathogens. GBS CPS and serine proteases CspA and ScpB hinder pathogen recognition by the host and thereby prevent phagocytic uptake and clearance of GBS.[18, 49, 58, 59] While CPS mimic host cells to prevent the recognition of GBS as a pathogen, serine proteases cleave peptides important in the signaling pathways of phagocytic recruitment and uptake of GBS. Evasion of oxidative stress from host-generated ROS by superoxide dismutase (SodA) also allows GBS to

persist *in vivo* (Figure 1.4).[18] GBS utilizes sensor kinases (Stk1 and CovS) to determine the appropriate toxins and virulence factors to express in various host environments and defend against the host's immune system.[18, 32]

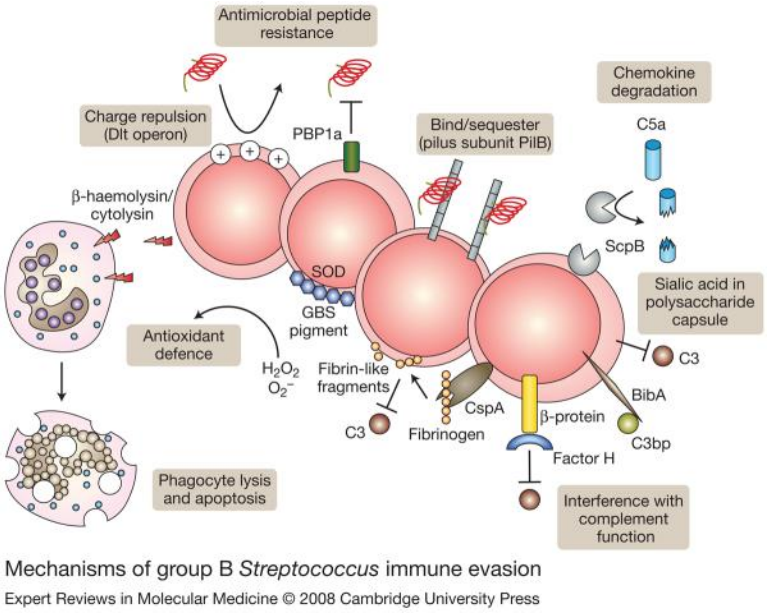


Figure 1. 4 Mechanisms of group B *Streptococcus* immune evasion. Group B *Streptococcus* (GBS) express several surface-expressed or secreted factors to evade host immune defenses and promote survival. The *Dlt* operon is responsible for increasing incorporation of D-alanine residues in cell-wall teichoic acids, thereby reducing electronegativity and affinity for cationic antimicrobial peptides. PBP1a and the pilB subunit of GBS pili also contribute to antimicrobial peptide resistance. ScpB, the sialic acid capsule, BibA, β protein and CspA all inhibit host clearance of GBS by interfering with complement components C5a, C3 and C3bp. SodA properties of the orange carotenoid pigment shield GBS from killing by phagocyte-generated reactive oxygen species. Alternatively, β-haemolysin/cytolysin can boost GBS survival by cytolytic or proapoptotic injury to host phagocytes. Abbreviations: BibA, GBS immunogenic bacterial adhesin; CspA, cell-surface protease A; PBP1a, penicillin-binding protein 1a; ScpB, C5a peptidase; SodA, superoxide dismutase. (Reproduced with permission from Maisey HC, Doran KS, Nizet V. Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. *Expert Reviews in Molecular Medicine*. 2008;10:e27. doi:10.1017/S1462399408000811.)

Biofilm formation is an important aspect of GBS pathogenesis that provides increased resistance to antimicrobial agents as well as host defenses.[60] GBS pili have been shown to be

important for the production of biofilm.[60] In a study conducted in Poland by Kaczorek, over 70% of isolates (n = 27) had the ability to produce biofilm, and gene sequencing of these isolates revealed the prevalence of virulence-related genes encoding for protease-resistance surface proteins (*rib*), proteins used for adherence (*bca*), and pore forming toxins (*cylE* and *cfb*).[61] A study by Boonyayatra showed a correlation between strains that exhibited antimicrobial resistance and produced biofilm.[62] Payot and Dramsi have demonstrated the importance of GBS capsule polysaccharide biosynthesis in mediating biofilm formation.[63, 64] Specifically, mutants missing either the *cpsE* gene, which is responsible for capsule formation, or the four *neuBCDA* genes, which are responsible for synthesis and modification of sialic acid residues, decreased the capacity of GBS to form biofilm *in vitro*.[64] Due to the relationship between GBS biofilm production and virulence, developing treatments or preventions that specifically target biofilms could lead to an avenue of viable therapeutics.

The capsular polysaccharide (CPS), part of the GBS capsule, is the most extensively studied GBS virulence factor.[18] GBS CPS mimic host glycans, which allows for bacterial evasion of host innate immune responses.[65] Due to this mimicry, the host immune response is less able to recognize GBS as a pathogen. GBS strains can be divided into serotypes based on CPS structure and polymerization. However, GBS CPS biosynthesis is similar across various strains, with the regulation, chain length, sialic acid synthesis, and oligosaccharide polymerization determined by sixteen genes of the *cps* loci.[65]

There are ten capsular serotypes of GBS that have been identified to date: Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. The CPS of all serotypes share several constituent monosaccharides, but each is distinct in the polysaccharide repeating unit and antigen response.[66] Conservation of an α-

D-Neu5Ac(2→3)-β-D-Gal motif is found across all serotypes, suggesting that this structural feature is important for virulence and immune invasion (Figure 1.5).[65, 66] A rhamnose residue distinguishes Type VIII varies from other serotypes.[67] The most recently identified GBS capsular based serotype is Type IX, which is most similar to Types V and VII. [68, 69] The repeat units and polymerization patterns of each known serotype are shown in Figure 1.6. [66, 70-72] [67-69, 73-77]

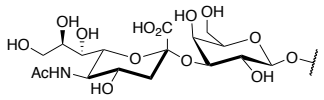


Figure 1. 5 Structural motif common to all GBS CPS.

Some GBS isolates do not belong to any of the ten unique capsular serotypes shown above. These strains are referred to as non-typeable because they do not react with any of the CPS antisera that are associated with capsular serotypes I-IX.[78] Non-typeable strains typically have a modified capsule type or produce an undetectable level of capsule. Since non-typeable strains are indistinguishable by their CPS, genetic methods of identification have been developed. These approaches have shown that some non-typeable isolates share genomic DNA with serotypeable isolates.[79] Additionally, surface protein expression has been used to distinguish between GBS isolates.[78] Non-typeable GBS strains account for less than 3% of colonizing strains in the United States but can still cause invasive disease (ca. 1.5% of invasive strains).[78-81] As CPS serve as a strategy used by GBS strains to evade the innate immune system, non-encapsulated strains must have developed alternative mechanisms for this evasion. Even though the prevalence of non-typeable GBS is low, discovering and understanding the evasion and virulence factors of this

subtype remains important because the development of GBS vaccines are often based on CPS. Alternatively, prevention of GBS infections by non-typeable isolates includes the development of protein-based vaccines that consist of a combination of antigens.[82]

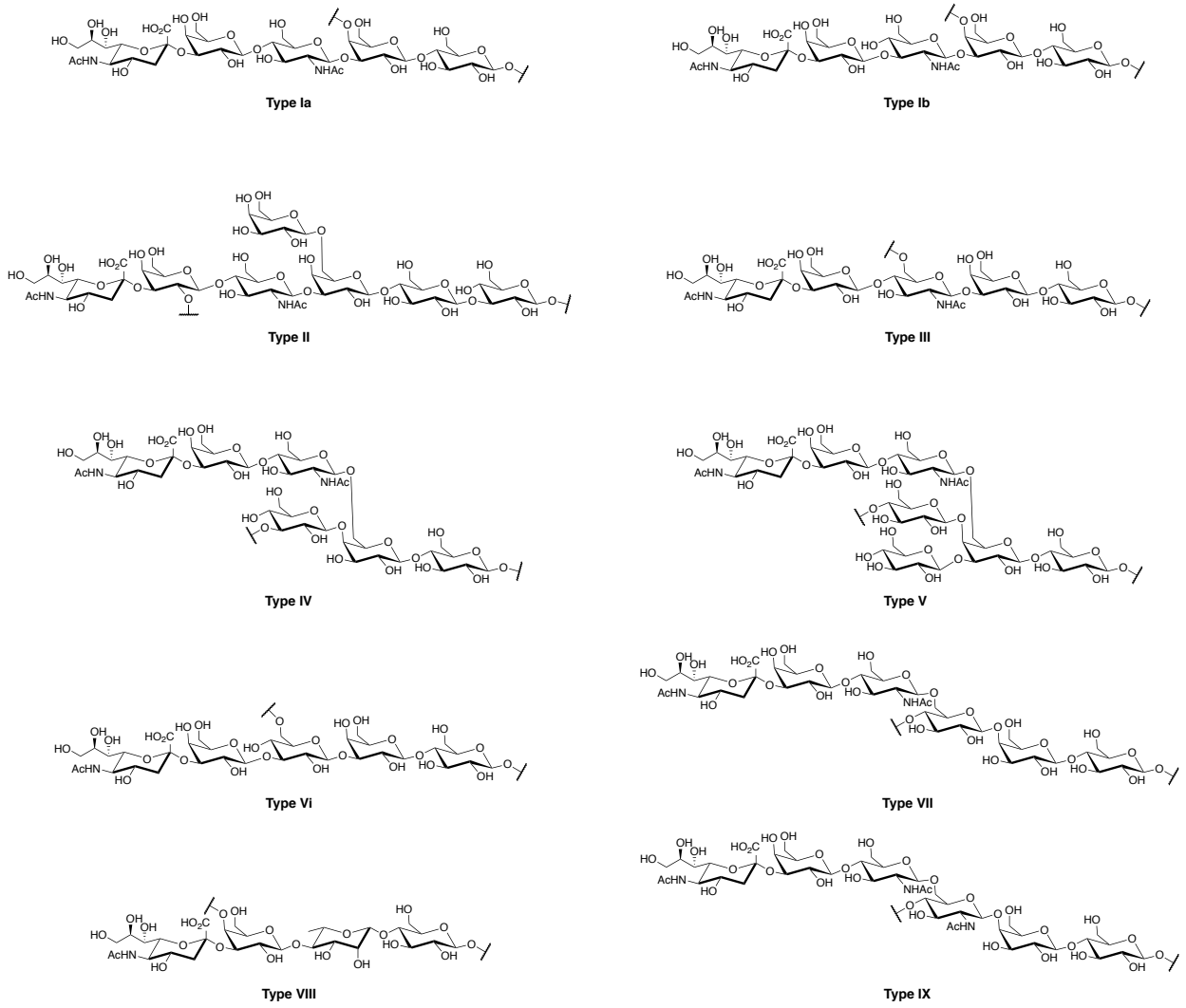


Figure 1. 6 Capsular Polysaccharide Repeat Units for GBS Serotypes

Methods for Serotyping and Other Classifications

GBS serotypes can be distinguished by several methods. The original method for serotyping was the immunoprecipitation test pioneered by Lancefield and is based on the formation of a precipitate after the cross-reaction of a protein antigen with its corresponding antibody.[83-86] The sensitivity of this method is dependent on the amount of antibody in the antiserum. In an effort to decrease the assay time of the immunoprecipitation test, immunofluorescence staining and counterimmunoelectrophoresis methods were developed for GBS.[86-88] Antibody-labeled reagent cells in slide coagglutination tests have also been used to identify and distinguish the serotypes of GBS.[86, 89, 90] To increase sensitivity and accessibility of materials needed for other serotyping methods, enzyme immunoassays have also been developed.[91, 92] The most commonly used serotyping method for GBS is latex agglutination tests that are based on the detection of antibodies specific for GBS CPS present in human serum.[93, 94]

Phenotypic assessment by CPS serotyping has been a long-standing method used in the description of GBS disease state and distribution. However, to better understand the expression of other virulence factors, especially those used by non-typeable strains, alternative methods have been developed to distinguish different strains. Early development of alternative typing methods included both genomic and molecular characterization methods.[95-98] Serotyping based on surface proteins instead of capsular polysaccharides has also proven to be useful in distinguishing between various protein serotypes of GBS.[78, 99, 100] Further, there are methods to analyze serovariant distribution, which is accounts for both the CPS serotype and expression of select surface proteins.[101] More recently, methods that focus on genotyping instead of serotyping have been developed. These include pulse-field gel electrophoresis, restriction endonuclease analysis, PCR-

based techniques, and sequence typing.[102-106] Studies dividing serovariants based on genes encoding for surface proteins have revealed that protein antigen serotyping and protein gene profiling can lead to inconsistencies in genetic and serotypic results.[99, 100] Similar studies to compare CPS serotyping and gene typing use sequences of the capsular polysaccharide synthesis (*cps*) gene clusters.[105] Gene typing allows for differentiation between GBS strains by sequence type. Multilocous sequence typing (MLST) is an expansion of initial gene typing methods that were based on the sequences of *cps* gene clusters. This sequence typing method involves nearly 500-base pair fragments of seven different housekeeping genes.[106] An online MLST database was established that offers investigators worldwide the ability to compare data from other geographically distinct regions, potentially providing insight into the epidemiology of GBS.[106, 107] More than 700 sequence types have been identified, and the majority of human isolates belong to six ancestral genotypes and strains.[19, 106, 108] MLST does not necessarily parallel capsular serotype, but subdivision of GBS strains by both serovariants and sequence types can increase the discriminatory power of typing systems to improve epidemiological and pathogenetic associations of GBS infections.[10, 99, 106]

Prevalence

A recent meta-analysis explored the prevalence of GBS colonization in both mothers and infants and the adverse outcomes associated with GBS.[109-118] While there are ten GBS capsular serotypes, five serotypes (Ia, Ib, II, III, and V) account for over 95% of GBS infections in infants (Figure 1.7).[114, 119] Serotype III is the most prevalent serotype in almost all countries and regions; however, serotype Ia is more prevalent than serotype III in South America.[114]

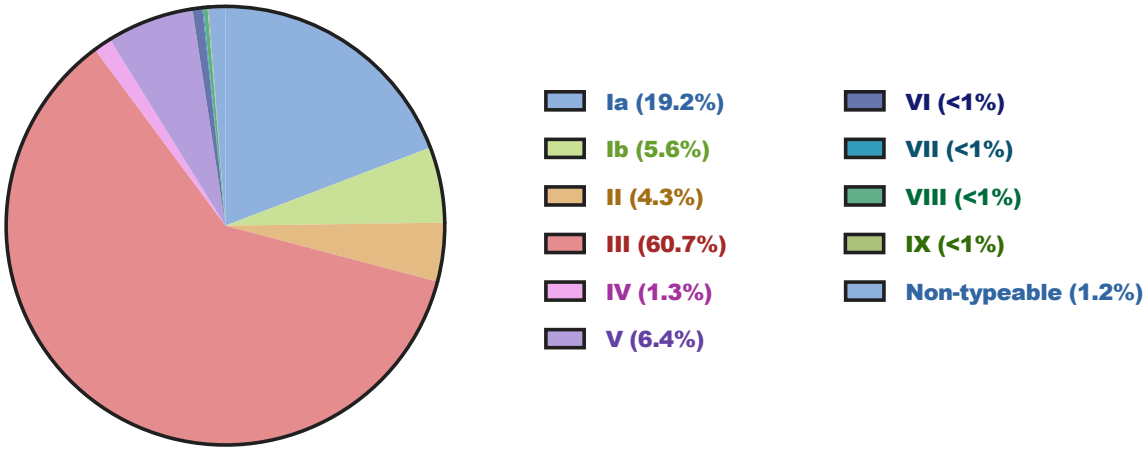


Figure 1. 7 Global distribution of GBS serotypes in invasive disease in young infants (N = 6500 isolates). Prevalence of GBS serotypes presented as percentage (number of cases). (Figure adapted from: Infant Group B *Streptococcal* Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis. 2017;65(suppl_2):S160-S172. doi:10.1093/cid/cix656)

Serotype distribution differs between EOD and LOD. Sereotype III is associated with 45% of EOD and 71% of LOD (Figure 1.8).[114] The other prominent serotypes, Types Ia, Ib, and V were more prevalent in EOD (20%, 8%, and 10%, respectively) than in LOD (14%, 6%, and 4%) (Figure 1.8).[114] Distribution of serotypes in maternal colonization may be linked to those seen in infant GBS diseases because vertical transmission of GBS from mother to infant would give rise to similarities in maternal colonization and infant infection.[113] Disease-causing serotypes are similarly distributed across different regions, and serotype distribution information can be used to inform the development of treatments and preventions because vaccines are often based on serotype.

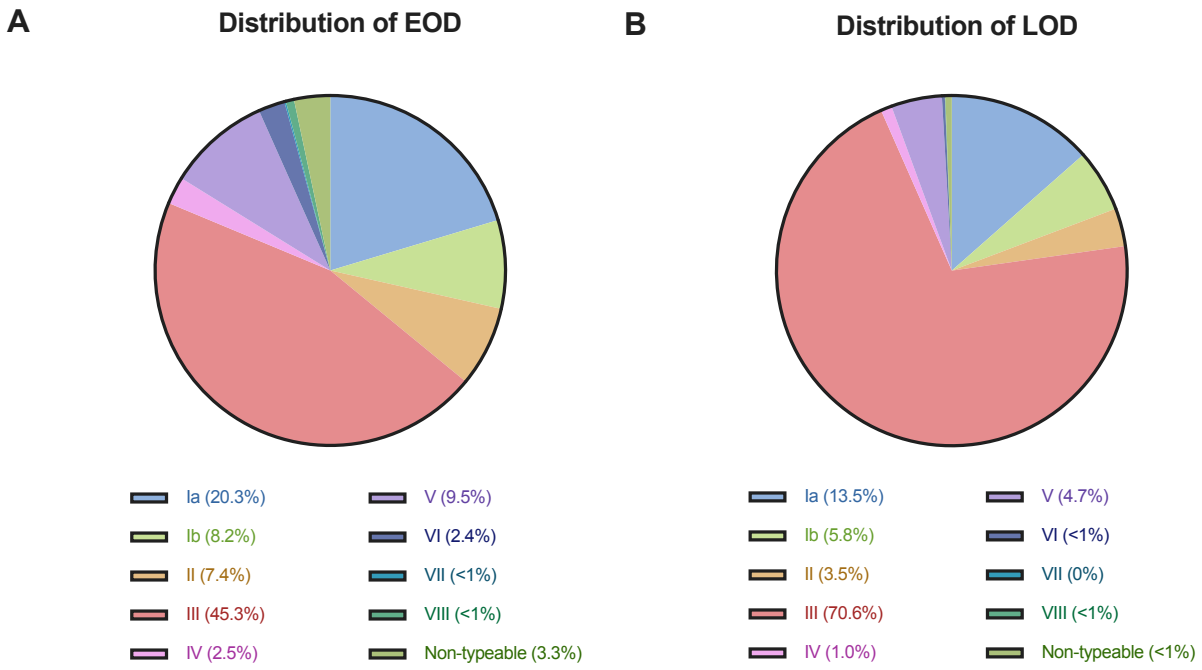


Figure 1. 8 Distribution of GBS serotypes for A) early onset GBS diseas and B) late onset GBS disease. (Figure adapted from: Infant Group B *Streptococcal* Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis. 2017;65(suppl_2):S160-S172. doi:10.1093/cid/cix656)

Treatments and Prevention

Because GBS is an important cause of perinatal morbidity and mortality, governing health bodies such as the Centers for Disease Control and Prevention (CDC), the American Academy of Pediatrics (AAP), the American College of Obstetricians and Gynecologists (ACOG), and the Royal College of Obstetricians and Gynecologists (RCOG) have issued statements suggesting strategies to prevent and treat GBS infection.[14, 15, 21, 120, 121] These strategies include prevention of vertical transmission by administering intrapartum antibiotic prophylaxis (IAP) to pregnant women who are colonized with GBS, which can prevent many perinatal infections.[121] Both risk-based and screening-based approaches have been used to identify women who should receive

IAP.[10] Common practice in industrialized countries includes prenatal screening to identify women who are colonized with GBS and should be given IAP during labor and delivery. The time-frame recommended for GBS screening is 35-37 weeks of gestation or 32-34 weeks for twins.[122] Identification of risk factors associated with GBS has also been used as a measure for determining the administration of IAP.[121]

Antibiotic prophylaxis given to mothers colonized with GBS has greatly reduced the incidence of EOD.[23, 123] In fact, since national guidelines for prevention and treatment of GBS were first released in the 1990s, incidences of EOD in the US has decreased by 80% (1.7 cases to < 0.4 cases per 1,000 live births).[15, 124] However, this approach is not always implemented in low and middle-income countries where perinatal health care fluctuates.[125] Cases of EOD have decreased with the use of IAP because it decreases maternal colonization and subsequent vertical transmission. Yet, IAP has had no effect on LOD or the incidences of stillbirth and prematurity related to GBS colonization because LOD is not directly correlated with maternal colonization and stillbirth and prematurity often occur before maternal screening for GBS.[18, 119]

The standard antibiotic given during prophylaxis is penicillin, with ampicillin commonly serving as an alternative (Table 1.3).[14, 121] Cefazolin is given to patients who have an allergy to penicillin and a low risk for anaphylaxis, angioedema, respiratory distress, or urticaria.[15] However, clindamycin is the antibiotic of choice for a patient with an allergy to penicillin and a high risk for anaphylaxis or respiratory stress,. Erythromycin could be used instead of clindamyin before revised guidelines were released in 2010 by the CDC and the National Center for Immunization and Respiratory Diseases.[124] Erythromycin has since been removed due to an increased resistance (25-35%) seen in several GBS strains.[15, 124, 126, 127] Because some strains of GBS have also

developed resistance to clindamycin (13-20%), it is recommended that GBS isolates be screened for resistance, and vancomycin is used as a last resort against resistant strains.[15, 124] These antibiotics are administered intravenously and given in doses aimed at achieving adequate levels in the fetal circulation and amniotic fluid.[124] Penicillin, ampicillin, and cefazolin are usually able to achieve high intra-amniotic concentrations. However, data suggests that clindamycin and vancomycin do not reach fetal tissues as readily.[124, 128-135] Because these alternatives have limitations in their pharmacokinetic profiles, alternative therapies need to be developed for women who react negatively to penicillin, ampicillin, or cefazolin. Additionally, many of the antibiotics used to treat GBS are broad spectrum and can influence the development of the infant microbiome by eliminating commensal bacteria. Thus, it is important to find alternative strategies to treat GBS. The structures of the antibiotics used to treat GBS are shown in Figure 1. 9.

Table 1. 3 Antibiotics Used to Treat GBS

Antibiotic	Administration	Resistance
Penicillin	Drug of choice	<2%[a]
Ampicillin	Alternative to penicillin	-
Cefazolin	Alternative for penicillin allergy	<1%
Clindamycin	High-risk of anaphylaxis	13-20%
Erythromycin	No longer used due to increased levels of resistance	25-35%
Vancomycin	Resistance to Clindamycin	<1%[b]

[a]reports listing intermediate or reduced susceptibility, but not entire resistance[138, 139] [b]only individual cases of vancomycin-resistant strains have been reported[140]

GBS resistance mechanisms to clindamycin and erythromycin are usually due to ribosomal modifications mediated by *erm* genes or upregulated efflux pumps encoded by *mef* genes.[136]

Resistance genes can be detected by PCR and studies have revealed that over 90% of resistant isolates (n = 88) carried *erm* genes and only 6% carried *mef* genes.[137, 136] No studies have been done to analyze the mechanisms associated with reduced susceptibility to penicillin, cefazolin, or vancomycin, but it is likely from similar mechanisms of ribosomal modification or up regulation of efflux pumps.

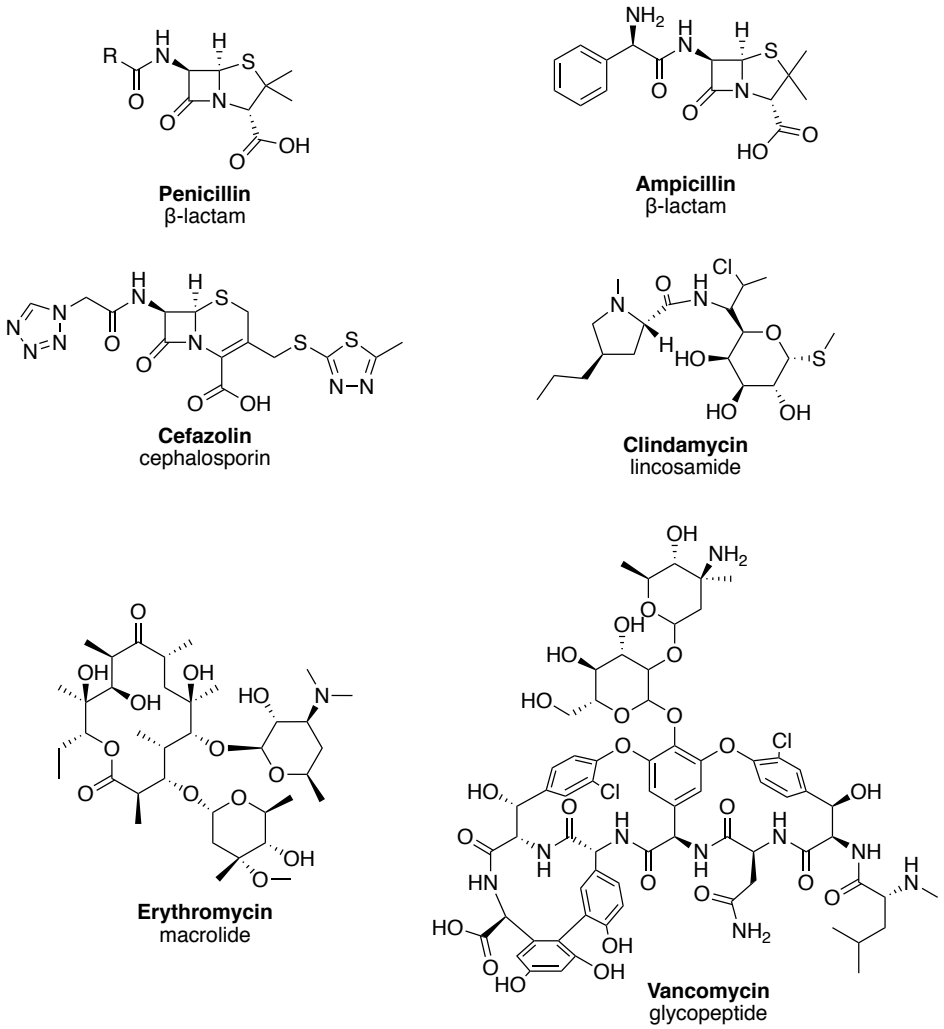


Figure 1. 9 Structures of Antibiotics Used to Treat Group B Strep

While antibiotics are given to treat GBS infections and to prevent transmission from mother to infant during labor and delivery, vaccination is another attractive preventative strategy. Although viral vaccines are more prevalent because bacterial infections can often be treated with antibiotics, several standard vaccines are available to prevent bacterial infections from pathogens such as tuberculosis, pertussis, and diphtheria.[141] The development of a successful GBS vaccine would rely on the production of protective antibodies that a mother could pass to her unborn child.[142] The transfer of antibodies from mother to fetus has been linked to infant protection against GBS infection.[143] CPS have structures similar to polysaccharides found in the host, which leads to lack of immunogenicity because the host is unable to recognize GBS as a foreign entity. As CPS play an important role in GBS virulence and antibodies to CPS have a protective role, vaccines using CPS as antigens have been developed and have entered clinical trials.[144-146] In the 1980s, the first GBS polysaccharide-vaccines were developed using type-specific polysaccharides for Type III CPS.[147] These initial studies demonstrated the feasibility of further developing CPS-based vaccines to protect neonates from GBS by maternal immunization. Other advancements in CPS-based vaccines included conjugation of proteins to increase immune response and utilization of a multivalent approach based on CPS of several serotypes.[144] Because five serotypes account for the majority of GBS disease, conjugate vaccines that incorporate polysaccharides from these serotypes could prevent roughly 95% of GBS disease in infants.[10, 119]

In addition to polysaccharide-based vaccines, protein-based vaccines have also gained considerable interest. New genomic information for both protein expression and CPS production has become available with the readily accessible analyses of complete genomes of varying GBS serotypes.[82] While CPS-based vaccines only have the ability to confer protection against the

serotypes included in the vaccine, protein-based vaccines could provide broad protection across all serotypes because they are based on ubiquitous GBS proteins.[148]

Several major pharmaceutical companies, including GlaxoSmithKline (GSK) and Pfizer, have developed CPS-based vaccines that have entered both phase I and II clinical trials (Table 1.4).[148] A protein-based vaccine developed by MinervaX has entered phase I clinical trials (Table 1.4).[148] As more vaccines are developed and make their way into clinical trials, it will be important to consider their use and efficacy in both high-income as well as low- and middle-income countries.[148, 149]

Table 1. 4 Overview of Vaccines in Development or Clinical Trials for GBS

	Clinical Trial Stage	**Type of Vaccine**
GSK	Phase I and II	Trivalent (Ia, Ib, III) CPS-CRM_{197}
GSK	Preclinical	Pentavalent (Ia, Ib, II, III, V) CPS-CRM_{197}
Pfizer	Early phase	Multi-valent CPS-CRM_{197}
MinervaX	Phase Ia and Ib	N-terminal domain fusion protein (Rib and AlpC) GBS-NN

Summary

Group B strep is an important pathogen during the neonatal and perinatal period, and mother and infant health are closely related during transmission. Even though there have been great advances in preventing the transmission of early onset disease, little is known about GBS conferral that causes late onset disease. GBS can asymptomatically colonize a healthy adult while

causing adverse pregnancy outcomes and invasive infection in infants. A better understanding of how this pathogen regulates virulence factors would aid in the development of treatment. Additionally, finding ways to utilize compounds produced by the host to develop protective and preventative strategies against GBS infections could be of great importance in overcoming the antimicrobial resistance of GBS.

References

1. Vornhagen, J.; Adams Waldorf, K. M.; Rajagopal, L., Perinatal Group B *Streptococcal* Infections: Virulence Factors, Immunity, and Prevention Strategies. *Trends Microbiol* **2017,** *25*, 919-931.

2. Lancefield, R. C., A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci. *J Exp Med* **1933,** *57*, 571-95.

3. Schuchat, A.; Wenger, J. D., Epidemiology of group B *streptococcal* disease. Risk factors, prevention strategies, and vaccine development. *Epidemiol Rev* **1994,** *16*, 374-402.

4. Simonsen, K. A.; Anderson-Berry, A. L.; Delair, S. F.; Davies, H. D., Early-onset neonatal sepsis. *Clin Microbiol Rev* **2014,** *27*, 21-47.

5. Keefe, G. P., *Streptococcus agalactiae* mastitis: a review. *Can Vet J* **1997,** *38*, 429-37.

6. Keefe, G., Update on control of Staphylococcus aureus and *Streptococcus agalactiae* for management of mastitis. *Vet Clin North Am Food Anim Pract* **2012,** *28*, 203-16.

7. Pinto, T. C.; Costa, N. S.; Correa, A. B.; de Oliveira, I. C.; de Mattos, M. C.; Rosado, A. S.; Benchetrit, L. C., Conjugative transfer of resistance determinants among human and bovine *Streptococcus agalactiae*. *Braz J Microbiol* **2014,** *45*, 785-9.

8. Maroney, M., *Streptococcus agalactiae*. In *Milk Money Fact Sheet 02* Pamela L. Ruegg, Dept. of Dairy Science at the University of Wisconsin2005.

9. Jimenez, E.; de Andres, J.; Manrique, M.; Pareja-Tobes, P.; Tobes, R.; Martinez-Blanch, J. F.; Codoner, F. M.; Ramon, D.; Fernandez, L.; Rodriguez, J. M., Metagenomic Analysis of Milk of Healthy and Mastitis-Suffering Women. *J Hum Lact* **2015,** *31*, 406-15.

10. Le Doare, K.; Heath, P. T., An overview of global GBS epidemiology. *Vaccine* **2013,** *31 Suppl 4*, D7-12.

11. Dermer, P.; Lee, C.; Eggert, J.; Few, B., A history of neonatal group B *streptococcus* with its related morbidity and mortality rates in the United States. *J Pediatr Nurs* **2004,** *19*, 357-63.

12. Le Doare, K.; O'Driscoll, M.; Turner, K.; Seedat, F.; Russell, N. J.; Seale, A. C.; Heath, P. T.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Kampmann, B.; Group, G. I. A. I., Intrapartum Antibiotic Chemoprophylaxis Policies for the Prevention of Group B *Streptococcal* Disease Worldwide: Systematic Review. *Clin Infect Dis* **2017,** *65*, S143-S151.

13. Sass, L., Group B *streptococcal* infections. *Pediatr Rev* **2012,** *33*, 219-24; quiz 224-5.

14. Prevention of Early-onset Neonatal Group B *Streptococcal* Disease: Green-top Guideline No. 36. *BJOG* **2017,** *124*, e280-e305.

15. American College of, O.; Gynecologists Committee on Obstetric, P., ACOG Committee Opinion No. 485: Prevention of early-onset group B *streptococcal* disease in newborns. *Obstet Gynecol* **2011,** *117*, 1019-27.

16. Hanley, J., Neonatal infections: group B *streptococcus*. *BMJ Clin Evid* **2008,** *2008*.

17. Madrid, L.; Seale, A. C.; Kohli-Lynch, M.; Edmond, K. M.; Lawn, J. E.; Heath, P. T.; Madhi, S. A.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Le Doare, K.; Rubens, C. E.; Saha, S. K.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Schrag, S.; Group, I. G. D. I., Infant Group B *Streptococcal* Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S160-S172.

18. Rajagopal, L., Understanding the regulation of Group B *Streptococcal* virulence factors. *Future Microbiol* **2009,** *4*, 201-21.

19. Kolter, J.; Henneke, P., Codevelopment of Microbiota and Innate Immunity and the Risk for Group B *Streptococcal* Disease. *Front Immunol* **2017,** *8*, 1497.

20. Melin, P., Neonatal group B *streptococcal* disease: from pathogenesis to preventive strategies. *Clin Microbiol Infect* **2011,** *17*, 1294-303.

21. Group B Strep (GBS). http://www.cdc.gov/groupbstrep/index.html (January 26),

22. Lin, F. Y.; Weisman, L. E.; Troendle, J.; Adams, K., Prematurity is the major risk factor for late-onset group B *streptococcus* disease. *J Infect Dis* **2003,** *188*, 267-71.

23. Schrag, S. J.; Zywicki, S.; Farley, M. M.; Reingold, A. L.; Harrison, L. H.; Lefkowitz, L. B.; Hadler, J. L.; Danila, R.; Cieslak, P. R.; Schuchat, A., Group B *streptococcal* disease in the era of intrapartum antibiotic prophylaxis. *N Engl J Med* **2000,** *342*, 15-20.

24. Boyer, K. M.; Vogel, L. C.; Gotoff, S. P.; Gadzala, C. A.; Stringer, J.; Maxted, W. R., Nosocomial transmission of bacteriophage type 7/11/12 group B *streptococci* in a special care nursery. *Am J Dis Child* **1980,** *134*, 964-6.

25. Glaser, P.; Rusniok, C.; Buchrieser, C.; Chevalier, F.; Frangeul, L.; Msadek, T.; Zouine, M.; Couve, E.; Lalioui, L.; Poyart, C.; Trieu-Cuot, P.; Kunst, F., Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol Microbiol* **2002,** *45*, 1499-513.

26. Doran, K. S.; Nizet, V., Molecular pathogenesis of neonatal group B *streptococcal* infection: no longer in its infancy. *Mol Microbiol* **2004,** *54*, 23-31.

27. Faralla, C.; Metruccio, M. M.; De Chiara, M.; Mu, R.; Patras, K. A.; Muzzi, A.; Grandi, G.; Margarit, I.; Doran, K. S.; Janulczyk, R., Analysis of two-component systems in group B *Streptococcus* shows that RgfAC and the novel FspSR modulate virulence and bacterial fitness. *MBio* **2014,** *5*, e00870-14.

28. Lamy, M. C.; Zouine, M.; Fert, J.; Vergassola, M.; Couve, E.; Pellegrini, E.; Glaser, P.; Kunst, F.; Msadek, T.; Trieu-Cuot, P.; Poyart, C., CovS/CovR of group B *streptococcus*: a two-component global regulatory system involved in virulence. *Mol Microbiol* **2004,** *54*, 1250-68.

29. Jiang, S. M.; Cieslewicz, M. J.; Kasper, D. L.; Wessels, M. R., Regulation of virulence by a two-component system in group B *streptococcus*. *J Bacteriol* **2005,** *187*, 1105-13.

30. Jiang, S. M.; Ishmael, N.; Dunning Hotopp, J.; Puliti, M.; Tissi, L.; Kumar, N.; Cieslewicz, M. J.; Tettelin, H.; Wessels, M. R., Variation in the group B *Streptococcus* CsrRS regulon and effects on pathogenicity. *J Bacteriol* **2008,** *190*, 1956-65.

31. Rajagopal, L.; Vo, A.; Silvestroni, A.; Rubens, C. E., Regulation of cytotoxin expression by converging eukaryotic-type and two-component signalling mechanisms in *Streptococcus agalactiae*. *Mol Microbiol* **2006,** *62*, 941-57.

32. Lin, W. J.; Walthers, D.; Connelly, J. E.; Burnside, K.; Jewell, K. A.; Kenney, L. J.; Rajagopal, L., Threonine phosphorylation prevents promoter DNA binding of the Group B *Streptococcus* response regulator CovR. *Mol Microbiol* **2009,** *71*, 1477-95.

33. Spellerberg, B.; Rozdzinski, E.; Martin, S.; Weber-Heynemann, J.; Lutticken, R., rgf encodes a novel two-component signal transduction system of *Streptococcus agalactiae*. *Infect Immun* **2002,** *70*, 2434-40.

34. Tettelin, H.; Masignani, V.; Cieslewicz, M. J.; Donati, C.; Medini, D.; Ward, N. L.; Angiuoli, S. V.; Crabtree, J.; Jones, A. L.; Durkin, A. S.; Deboy, R. T.; Davidsen, T. M.; Mora, M.; Scarselli, M.; Margarit y Ros, I.; Peterson, J. D.; Hauser, C. R.; Sundaram, J. P.; Nelson, W. C.; Madupu, R.; Brinkac, L. M.; Dodson, R. J.; Rosovitz, M. J.; Sullivan, S. A.; Daugherty, S. C.; Haft, D. H.; Selengut, J.; Gwinn, M. L.; Zhou, L.; Zafar, N.; Khouri, H.; Radune, D.; Dimitrov, G.; Watkins, K.; O'Connor, K. J.; Smith, S.; Utterback, T. R.; White, O.; Rubens, C. E.; Grandi, G.; Madoff, L. C.; Kasper, D. L.; Telford, J. L.; Wessels, M. R.; Rappuoli, R.; Fraser, C. M., Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". *Proc Natl Acad Sci U S A* **2005,** *102*, 13950-5.

35. Poyart, C.; Lamy, M. C.; Boumaila, C.; Fiedler, F.; Trieu-Cuot, P., Regulation of D-alanyl-lipoteichoic acid biosynthesis in *Streptococcus agalactiae* involves a novel two-component regulatory system. *J Bacteriol* **2001,** *183*, 6324-34.

36. Poyart, C.; Pellegrini, E.; Marceau, M.; Baptista, M.; Jaubert, F.; Lamy, M. C.; Trieu-Cuot, P., Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. *Mol Microbiol* **2003,** *49*, 1615-25.

37. Quach, D.; van Sorge, N. M.; Kristian, S. A.; Bryan, J. D.; Shelver, D. W.; Doran, K. S., The CiaR response regulator in group B *Streptococcus* promotes intracellular survival and resistance to innate immune defenses. *J Bacteriol* **2009,** *191*, 2023-32.

38. Mu, R.; Cutting, A. S.; Del Rosario, Y.; Villarino, N.; Stewart, L.; Weston, T. A.; Patras, K. A.; Doran, K. S., Identification of CiaR Regulated Genes That Promote Group B *Streptococcal* Virulence and Interaction with Brain Endothelial Cells. *Plos One* **2016,** *11*, e0153891.

39. Ulrich, L. E.; Koonin, E. V.; Zhulin, I. B., One-component systems dominate signal transduction in prokaryotes. *Trends Microbiol* **2005,** *13*, 52-6.

40. Kreikemeyer, B.; McIver, K. S.; Podbielski, A., Virulence factor regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions. *Trends Microbiol* **2003,** *11*, 224-32.

41. Tamura, G. S.; Kuypers, J. M.; Smith, S.; Raff, H.; Rubens, C. E., Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. *Infect Immun* **1994,** *62*, 2450-8.

42. Buscetta, M.; Papasergi, S.; Firon, A.; Pietrocola, G.; Biondo, C.; Mancuso, G.; Midiri, A.; Romeo, L.; Teti, G.; Speziale, P.; Trieu-Cuot, P.; Beninati, C., FbsC, a novel fibrinogen-

binding protein, promotes *Streptococcus agalactiae*-host cell interactions. *J Biol Chem* **2014,** *289*, 21003-21015.

43. Spellerberg, B.; Rozdzinski, E.; Martin, S.; Weber-Heynemann, J.; Schnitzler, N.; Lutticken, R.; Podbielski, A., Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. *Infect Immun* **1999,** *67*, 871-8.

44. Wang, N. Y.; Patras, K. A.; Seo, H. S.; Cavaco, C. K.; Rosler, B.; Neely, M. N.; Sullam, P. M.; Doran, K. S., Group B *streptococcal* serine-rich repeat proteins promote interaction with fibrinogen and vaginal colonization. *J Infect Dis* **2014,** *210*, 982-91.

45. Seo, H. S.; Xiong, Y. Q.; Sullam, P. M., Role of the serine-rich surface glycoprotein Srr1 of *Streptococcus agalactiae* in the pathogenesis of infective endocarditis. *Plos One* **2013,** *8*, e64204.

46. Sheen, T. R.; Jimenez, A.; Wang, N. Y.; Banerjee, A.; van Sorge, N. M.; Doran, K. S., Serine-rich repeat proteins and pili promote *Streptococcus agalactiae* colonization of the vaginal tract. *J Bacteriol* **2011,** *193*, 6834-42.

47. Santi, I.; Scarselli, M.; Mariani, M.; Pezzicoli, A.; Masignani, V.; Taddei, A.; Grandi, G.; Telford, J. L.; Soriani, M., BibA: a novel immunogenic bacterial adhesin contributing to group B *Streptococcus* survival in human blood. *Mol Microbiol* **2007,** *63*, 754-67.

48. Dramsi, S.; Caliot, E.; Bonne, I.; Guadagnini, S.; Prevost, M. C.; Kojadinovic, M.; Lalioui, L.; Poyart, C.; Trieu-Cuot, P., Assembly and role of pili in group B *streptococci. Mol Microbiol* **2006,** *60*, 1401-13.

49. Maisey, H. C.; Doran, K. S.; Nizet, V., Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. *Expert Rev Mol Med* **2008,** *10*, e27.

50. Tenenbaum, T.; Spellerberg, B.; Adam, R.; Vogel, M.; Kim, K. S.; Schroten, H., *Streptococcus agalactiae* invasion of human brain microvascular endothelial cells is promoted by the laminin-binding protein Lmb. *Microbes Infect* **2007,** *9*, 714-20.

51. Gutekunst, H.; Eikmanns, B. J.; Reinscheid, D. J., The novel fibrinogen-binding protein FbsB promotes *Streptococcus agalactiae* invasion into epithelial cells. *Infect Immun* **2004,** *72*, 3495-504.

52. Baron, M. J.; Bolduc, G. R.; Goldberg, M. B.; Auperin, T. C.; Madoff, L. C., Alpha C protein of group B *Streptococcus* binds host cell surface glycosaminoglycan and enters cells by an actin-dependent mechanism. *J Biol Chem* **2004,** *279*, 24714-23.

53. Baron, M. J.; Filman, D. J.; Prophete, G. A.; Hogle, J. M.; Madoff, L. C., Identification of a glycosaminoglycan binding region of the alpha C protein that mediates entry of group B *Streptococci* into host cells. *J Biol Chem* **2007,** *282*, 10526-36.

54. Bolduc, G. R.; Baron, M. J.; Gravekamp, C.; Lachenauer, C. S.; Madoff, L. C., The alpha C protein mediates internalization of group B *Streptococcus* within human cervical epithelial cells. *Cell Microbiol* **2002,** *4*, 751-8.

55. Hensler, M. E.; Quach, D.; Hsieh, C. J.; Doran, K. S.; Nizet, V., CAMP factor is not essential for systemic virulence of Group B *Streptococcus*. *Microb Pathog* **2008,** *44*, 84-8.

56. Soriani, M.; Santi, I.; Taddei, A.; Rappuoli, R.; Grandi, G.; Telford, J. L., Group B *Streptococcus* crosses human epithelial cells by a paracellular route. *J Infect Dis* **2006,** *193*, 241-50.

57. Maisey, H. C.; Quach, D.; Hensler, M. E.; Liu, G. Y.; Gallo, R. L.; Nizet, V.; Doran, K. S., A group B *streptococcal* pilus protein promotes phagocyte resistance and systemic virulence. *FASEB J* **2008,** *22*, 1715-24.

58. Bohnsack, J. F.; Widjaja, K.; Ghazizadeh, S.; Rubens, C. E.; Hillyard, D. R.; Parker, C. J.; Albertine, K. H.; Hill, H. R., A role for C5 and C5a-ase in the acute neutrophil response to group B *streptococcal* infections. *J Infect Dis* **1997,** *175*, 847-55.

59. Takahashi, S.; Nagano, Y.; Nagano, N.; Hayashi, O.; Taguchi, F.; Okuwaki, Y., Role of C5a-ase in group B *streptococcal* resistance to opsonophagocytic killing. *Infect Immun* **1995,** *63*, 4764-9.

60. Rosini, R.; Margarit, I., Biofilm formation by *Streptococcus agalactiae*: influence of environmental conditions and implicated virulence factors. *Front Cell Infect Microbiol* **2015,** *5*, 6.

61. Kaczorek, E.; Malaczewska, J.; Wojcik, R.; Siwicki, A. K., Biofilm production and other virulence factors in *Streptococcus spp*. isolated from clinical cases of bovine mastitis in Poland. *BMC Vet Res* **2017,** *13*, 398.

62. Boonyayatra, S.; Pata, P.; Nakharuthai, P.; Chaisri, W., Antimicrobial Resistance of Biofilm-Forming *Streptococcus agalactiae* Isolated from Bovine Mastitis. *J Vet Sci Technol* **2016,** *7*.

63. Chuzeville, S.; Dramsi, S.; Madec, J. Y.; Haenni, M.; Payot, S., Antigen I/II encoded by integrative and conjugative elements of *Streptococcus agalactiae* and role in biofilm formation. *Microb Pathog* **2015,** *88*, 1-9.

64. Xia, F. D.; Mallet, A.; Caliot, E.; Gao, C.; Trieu-Cuot, P.; Dramsi, S., Capsular polysaccharide of Group B *Streptococcus* mediates biofilm formation in the presence of human plasma. *Microbes Infect* **2015,** *17*, 71-6.

65. Segura, M., Fisher scientific award lecture - the capsular polysaccharides of Group B *Streptococcus* and *Streptococcus suis* differently modulate bacterial interactions with dendritic cells. *Can J Microbiol* **2012,** *58*, 249-60.

66. Cieslewicz, M. J.; Chaffin, D.; Glusman, G.; Kasper, D.; Madan, A.; Rodrigues, S.; Fahey, J.; Wessels, M. R.; Rubens, C. E., Structural and genetic diversity of group B *streptococcus* capsular polysaccharides. *Infect Immun* **2005,** *73*, 3096-103.

67. Kogan, G.; Uhrin, D.; Brisson, J. R.; Paoletti, L. C.; Blodgett, A. E.; Kasper, D. L.; Jennings, H. J., Structural and immunochemical characterization of the type VIII group B *Streptococcus* capsular polysaccharide. *J Biol Chem* **1996,** *271*, 8786-90.

68. Berti, F.; Campisi, E.; Toniolo, C.; Morelli, L.; Crotti, S.; Rosini, R.; Romano, M. R.; Pinto, V.; Brogioni, B.; Torricelli, G.; Janulczyk, R.; Grandi, G.; Margarit, I., Structure of the type IX group B *Streptococcus* capsular polysaccharide and its evolutionary relationship with types V and VII. *J Biol Chem* **2014,** *289*, 23437-48.

69. Slotved, H. C.; Kong, F.; Lambertsen, L.; Sauer, S.; Gilbert, G. L., Serotype IX, a Proposed New *Streptococcus agalactiae* Serotype. *J Clin Microbiol* **2007,** *45*, 2929-36.

70. Jennings, H. J.; Katzenellenbogen, E.; Lugowski, C.; Kasper, D. L., Structure of native polysaccharide antigens of type Ia and type Ib group B *Streptococcus*. *Biochemistry* **1983,** *22*, 1258-64.

71. Jennings, H. J.; Lugowski, C.; Kasper, D. L., Conformational aspects critical to the immunospecificity of the type III group B *streptococcal* polysaccharide. *Biochemistry* **1981,** *20*, 4511-8.

72. Wessels, M. R.; Pozsgay, V.; Kasper, D. L.; Jennings, H. J., Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B *Streptococcus*. A revised structure for the type III group B *streptococcal* polysaccharide antigen. *J Biol Chem* **1987,** *262*, 8262-7.

73. Fabio, J. L. D.; Michon, F.; Brisson, J.-R.; Jennings, H. J.; Wessels, M. R.; Benedí, V.-J.; Kasper, D. L., Structure of the capsular polysaccharide antigen of type IV group B *Streptococcus*. *Can J Chem* **1989,** *67*, 877-882.

74. Jennings, H. J.; Rosell, K. G.; Katzenellenbogen, E.; Kasper, D. L., Structural determination of the capsular polysaccharide antigen of type II group B *Streptococcus*. *J Biol Chem* **1983,** *258*, 1793-8.

75. Wessels, M. R.; DiFabio, J. L.; Benedi, V. J.; Kasper, D. L.; Michon, F.; Brisson, J. R.; Jelinkova, J.; Jennings, H. J., Structural determination and immunochemical characterization of the type V group B *Streptococcus* capsular polysaccharide. *J Biol Chem* **1991,** *266*, 6714-9.

76. Kogan, G.; Brisson, J. R.; Kasper, D. L.; von Hunolstein, C.; Orefici, G.; Jennings, H. J., Structural elucidation of the novel type VII group B *Streptococcus* capsular polysaccharide by high resolution NMR spectroscopy. *Carbohydr Res* **1995,** *277*, 1-9.

77. von Hunolstein, C.; D'Ascenzi, S.; Wagner, B.; Jelinkova, J.; Alfarone, G.; Recchia, S.; Wagner, M.; Orefici, G., Immunochemistry of capsular type polysaccharide and virulence properties of type VI *Streptococcus agalactiae* (group B *streptococci*). *Infect Immun* **1993,** *61*, 1272-80.

78. Ramaswamy, S. V.; Ferrieri, P.; Flores, A. E.; Paoletti, L. C., Molecular characterization of nontypeable group B streptococcus. *J Clin Microbiol* **2006,** *44*, 2398-403.

79. Benson, J. A.; Flores, A. E.; Baker, C. J.; Hillier, S. L.; Ferrieri, P., Improved methods for typing nontypeable isolates of group B *streptococci*. *Int J Med Microbiol* **2002,** *292*, 37-42.

80. Amundson, N. R.; Flores, A. E.; Hillier, S. L.; Baker, C. J.; Ferrieri, P., DNA macrorestriction analysis of nontypeable group B *streptococcal* isolates: clonal evolution of nontypeable and type V isolates. *J Clin Microbiol* **2005,** *43*, 572-6.

81. Ferrieri, P.; Baker, C. J.; Hillier, S. L.; Flores, A. E., Diversity of surface protein expression in group B *streptococcal* colonizing & invasive isolates. *Indian J Med Res* **2004,** *119 Suppl*, 191-6.

82. Maione, D.; Margarit, I.; Rinaudo, C. D.; Masignani, V.; Mora, M.; Scarselli, M.; Tettelin, H.; Brettoni, C.; Iacobini, E. T.; Rosini, R.; D'Agostino, N.; Miorin, L.; Buccato, S.; Mariani, M.; Galli, G.; Nogarotto, R.; Nardi-Dei, V.; Vegni, F.; Fraser, C.; Mancuso, G.; Teti, G.; Madoff, L. C.; Paoletti, L. C.; Rappuoli, R.; Kasper, D. L.; Telford, J. L.; Grandi, G., Identification of a universal Group B *streptococcus* vaccine by multiple genome screen. *Science* **2005,** *309*, 148-50.

83. Lancefield, R. C., Microprecipitin-technic for classifying Hemolytic *Streptococci*, and Improved Methods for Producing Antisera. *Proc Soc Exp Biol Med* **1938,** *38*, 473-478.

84. Lancefield, R. C.; McCarty, M.; Everly, W. N., Multiple mouse-protective antibodies directed against group B *streptococci*. Special reference to antibodies effective against protein antigens. *J Exp Med* **1975,** *142*, 165-79.

85. Wilkinson, H. W.; Moody, M. D., Serological relationships of type I antigens of group B *streptococci*. *J Bacteriol* **1969,** *97*, 629-34.

86. Triscott, M. X.; Davis, G. H., A comparison of four methods for the serotyping of group B *streptococci*. *Aust J Exp Biol Med Sci* **1979,** *57*, 521-7.

87. Hill, H. R.; Riter, M. E.; Menge, S. K.; Johnson, D. R.; Matsen, J. M., Rapid identification of group B *streptococci* by counterimmunoelectrophoresis. *J Clin Microbiol* **1975,** *1*, 188-91.

88. Romero, R.; Wilkinson, H. W., Identification of group B *streptococci* by immunofluorescence staining. *Appl Microbiol* **1974,** *28*, 199-204.

89. Kirkegaard, M. K.; Field, C. R., Rapid slide coagglutination test for identifying and typing group B *streptococci*. *J Clin Microbiol* **1977,** *6*, 266-70.

90. Hakansson, S.; Burman, L. G.; Henrichsen, J.; Holm, S. E., Novel coagglutination method for serotyping group B *streptococci*. *J Clin Microbiol* **1992,** *30*, 3268-9.

91. Holm, S. E.; Hakansson, S., A simple and sensitive enzyme immunoassay for determination of soluble type-specific polysaccharide from group B *streptococci*. *J Immunol Methods* **1988,** *106*, 89-94.

92. Arakere, G.; Flores, A. E.; Ferrieri, P.; Frasch, C. E., Inhibition enzyme-linked immunosorbent assay for serotyping of group B *streptococcal* isolates. *J Clin Microbiol* **1999,** *37*, 2564-7.

93. Zuerlein, T. J.; Christensen, B.; Hall, R. T., Latex agglutination detection of group-B *streptococcal* inoculum in urine. *Diagn Microbiol Infect Dis* **1991,** *14*, 191-4.

94. Brigtsen, A. K.; Dedi, L.; Melby, K. K.; Holberg-Petersen, M.; Radtke, A.; Lyng, R. V.; Andresen, L. L.; Jacobsen, A. F.; Fugelseth, D.; Whitelaw, A., Comparison of PCR and serotyping of Group B *Streptococcus* in pregnant women: the Oslo GBS-study. *J Microbiol Methods* **2015,** *108*, 31-5.

95. Colman, G., Typing of *Streptococcus agalactiae* (Lancefield group B). *Eur J Clin Microbiol Infect Dis* **1988,** *7*, 226-31.

96. Musser, J. M.; Mattingly, S. J.; Quentin, R.; Goudeau, A.; Selander, R. K., Identification of a high-virulence clone of type III *Streptococcus agalactiae* (group B *Streptococcus*) causing invasive neonatal disease. *Proc Natl Acad Sci U S A* **1989,** *86*, 4731-5.

97. Denning, D. W.; Baker, C. J.; Troup, N. J.; Tompkins, L. S., Restriction endonuclease analysis of human and bovine group B *streptococci* for epidemiologic study. *J Clin Microbiol* **1989,** *27*, 1352-6.

98. Fasola, E.; Livdahl, C.; Ferrieri, P., Molecular analysis of multiple isolates of the major serotypes of group B *streptococci*. *J Clin Microbiol* **1993,** *31*, 2616-20.

99. Kong, F.; Gowan, S.; Martin, D.; James, G.; Gilbert, G. L., Molecular profiles of group B *streptococcal* surface protein antigen genes: relationship to molecular serotypes. *J Clin Microbiol* **2002,** *40*, 620-6.

100. Radtke, A.; Kong, F.; Bergh, K.; Lyng, R. V.; Ko, D.; Gilbert, G. L., Identification of surface proteins of group B *streptococci*: serotyping versus genotyping. *J Microbiol Methods* **2009,** *78*, 363-5.

101. Kvam, A. I.; Efstratiou, A.; Bevanger, L.; Cookson, B. D.; Marticorena, I. F.; George, R. C.; Maeland, J. A., Distribution of serovariants of group B *streptococci* in isolates from England and Norway. *J Med Microbiol* **1995,** *42*, 246-50.

102. Rolland, K.; Marois, C.; Siquier, V.; Cattier, B.; Quentin, R., Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-field gel electrophoresis and hylB gene analysis. *J Clin Microbiol* **1999,** *37*, 1892-8.

103. Nagano, Y.; Nagano, N.; Takahashi, S.; Murono, K.; Fujita, K.; Taguchi, F.; Okuwaki, Y., Restriction endonuclease digest patterns of chromosomal DNA from group B β-haemolytic *streptococci*. *J Med Microbiol* **1991,** *35*, 297-303.

104. Ke, D.; Menard, C.; Picard, F. J.; Boissinot, M.; Ouellette, M.; Roy, P. H.; Bergeron, M. G., Development of conventional and real-time PCR assays for the rapid detection of group B *streptococci*. *Clin Chem* **2000,** *46*, 324-31.

105. Kong, F.; Gowan, S.; Martin, D.; James, G.; Gilbert, G. L., Serotype identification of group B *streptococci* by PCR and sequencing. *J Clin Microbiol* **2002,** *40*, 216-26.

106. Jones, N.; Bohnsack, J. F.; Takahashi, S.; Oliver, K. A.; Chan, M. S.; Kunst, F.; Glaser, P.; Rusniok, C.; Crook, D. W.; Harding, R. M.; Bisharat, N.; Spratt, B. G., Multilocus sequence typing system for group B *streptococcus*. *J Clin Microbiol* **2003,** *41*, 2530-6.

107. Jolley, K. A.; CJ, M. M., *Streptococcus agalactiae* MLST Databases. https://pubmlst.org/sagalactiae/ University of Oxford.

108. Da Cunha, V.; Davies, M. R.; Douarre, P. E.; Rosinski-Chupin, I.; Margarit, I.; Spinali, S.; Perkins, T.; Lechat, P.; Dmytruk, N.; Sauvage, E.; Ma, L.; Romi, B.; Tichit, M.; Lopez-Sanchez, M. J.; Descorps-Declere, S.; Souche, E.; Buchrieser, C.; Trieu-Cuot, P.; Moszer,

I.; Clermont, D.; Maione, D.; Bouchier, C.; McMillan, D. J.; Parkhill, J.; Telford, J. L.; Dougan, G.; Walker, M. J.; Consortium, D.; Holden, M. T. G.; Poyart, C.; Glaser, P., *Streptococcus agalactiae* clones infecting humans were selected and fixed through the extensive use of tetracycline. *Nat Commun* **2014,** *5*, 4544.

109. Hall, J.; Adams, N. H.; Bartlett, L.; Seale, A. C.; Lamagni, T.; Bianchi-Jassir, F.; Lawn, J. E.; Baker, C. J.; Cutland, C.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Gravett, M. G., Maternal Disease With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S112-S124.

110. Seale, A. C.; Blencowe, H.; Bianchi-Jassir, F.; Embleton, N.; Bassat, Q.; Ordi, J.; Menendez, C.; Cutland, C.; Briner, C.; Berkley, J. A.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Meulen, A. S.; Vekemans, J.; Madhi, S. A., Stillbirth With Group B *Streptococcus* Disease Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S125-S132.

111. Bianchi-Jassir, F.; Seale, A. C.; Kohli-Lynch, M.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Rubens, C. E., Preterm Birth Associated With Group B *Streptococcus* Maternal Colonization Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S133-S142.

112. Le Doare, K.; O'Driscoll, M.; Turner, K.; Seedat, F.; Russell, N. J.; Seale, A. C.; Heath, P. T.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Kampmann, B.; Group, G. B. S. I. A. I., Intrapartum Antibiotic Chemoprophylaxis Policies for the Prevention of Group B *Streptococcal* Disease Worldwide: Systematic Review. *Clin Infect Dis* **2017,** *65*, S143-S151.

113. Russell, N. J.; Seale, A. C.; O'Driscoll, M.; O'Sullivan, C.; Bianchi-Jassir, F.; Gonzalez-Guarin, J.; Lawn, J. E.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Saha, S. K.; Ip, M.; Group, G. B. S. M. C. I., Maternal Colonization With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S100-S111.

114. Madrid, L.; Seale, A. C.; Kohli-Lynch, M.; Edmond, K. M.; Lawn, J. E.; Heath, P. T.; Madhi, S. A.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Le Doare, K.; Rubens, C. E.; Saha, S. K.; Sobanjo-ter Meulen, A.; Vekemans, J.; Schrag, S.; Agarwal, R.; da Silva, A. R. A.; Bassat, Q.; Berkley, J. A.; Dangor, Z.; Dhaded, S.; Giannoni, E.; Hammoud, M.; Kobayahsi, M.; O'Sullivan, C.; Sakata, H.; Sridhar, S.; Sigaúque, B.; Tyrrell, G.; Paul, V., Infant Group B *Streptococcal* Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S160-S172.

115. Tann, C. J.; Martinello, K. A.; Sadoo, S.; Lawn, J. E.; Seale, A. C.; Vega-Poblete, M.; Russell, N. J.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Heath, P. T.; Group, G. B. S. N. E. I., Neonatal Encephalopathy With Group B *Streptococcal* Disease Worldwide: Systematic Review, Investigator Group Datasets, and Meta-analysis. *Clin Infect Dis* **2017,** *65*, S173-S189.

116. Kohli-Lynch, M.; Russell, N. J.; Seale, A. C.; Dangor, Z.; Tann, C. J.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; O'Sullivan, C.; Nakwa, F.; Ben Hamouda, H.; Soua, H.; Giorgakoudi, K.; Ladhani, S.; Lamagni, T.; Rattue, H.; Trotter, C.; Lawn, J. E., Neurodevelopmental Impairment in Children After Group B *Streptococcal* Disease Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S190-S199.

117. Seale, A. C.; Bianchi-Jassir, F.; Russell, N. J.; Kohli-Lynch, M.; Tann, C. J.; Hall, J.; Madrid, L.; Blencowe, H.; Cousens, S.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S. J.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Lawn, J. E., Estimates of the Burden of Group B *Streptococcal* Disease Worldwide for Pregnant Women, Stillbirths, and Children. *Clin Infect Dis* **2017,** *65*, S200-S219.

118. Lawn, J. E.; Bianchi-Jassir, F.; Russell, N. J.; Kohli-Lynch, M.; Tann, C. J.; Hall, J.; Madrid, L.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Heath, P. T.; Ip, M.; Le Doare, K.; Madhi, S. A.; Rubens, C. E.; Saha, S. K.; Schrag, S.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Seale, A. C., Group B *Streptococcal* Disease Worldwide for Pregnant Women, Stillbirths, and Children: Why, What, and How to Undertake Estimates? *Clin Infect Dis* **2017,** *65*, S89-S99.

119. Edmond, K. M.; Kortsalioudaki, C.; Scott, S.; Schrag, S. J.; Zaidi, A. K. M.; Cousens, S.; Heath, P. T., Group B *streptococcal* disease in infants aged younger than 3 months: systematic review and meta-analysis. *Lancet* **2012,** *379*, 547-556.

120. Revised Guidelines for Prevention of Early-onset Group B *Streptococcal* (GBS) Infection. *Pediatrics* **1997,** *99*, 489-496.

121. Centers for Disease Control and Prevention. Prevention of perinatal group B *streptococcal* disease: a public health perspective. *MMWR Recomm Rep* **1996,** *45*, 1-24.

122. Prevention of Early-onset Neonatal Group B *Streptococcal* Disease. *BJOG: An International Journal of Obstetrics & Gynaecology* **2017,** *124*, e280-e305.

123. Schrag, S.; Gorwitz, R.; Fultz-Butts, K.; Schuchat, A., Prevention of perinatal group B *streptococcal* disease. Revised guidelines from CDC. *MMWR Recomm Rep* **2002,** *51*, 1-22.

124. Verani, J. R.; McGee, L.; Schrag, S. J.; Division of Bacterial Diseases, N. C. f. I.; Respiratory Diseases, C. f. D. C.; Prevention, Prevention of perinatal group B *streptococcal* disease-revised guidelines from CDC, 2010. *MMWR Recomm Rep* **2010,** *59*, 1-36.

125. Bhutta, Z. A.; Darmstadt, G. L.; Hasan, B. S.; Haws, R. A., Community-based interventions for improving perinatal and neonatal health outcomes in developing countries: a review of the evidence. *Pediatrics* **2005,** *115*, 519-617.

126. Borchardt, S. M.; DeBusscher, J. H.; Tallman, P. A.; Manning, S. D.; Marrs, C. F.; Kurzynski, T. A.; Foxman, B., Frequency of antimicrobial resistance among invasive and colonizing Group B *streptococcal* isolates. *BMC Infect Dis* **2006,** *6*, 57.

127. Castor, M. L.; Whitney, C. G.; Como-Sabetti, K.; Facklam, R. R.; Ferrieri, P.; Bartkus, J. M.; Juni, B. A.; Cieslak, P. R.; Farley, M. M.; Dumas, N. B.; Schrag, S. J.; Lynfield, R., Antibiotic resistance patterns in invasive group B *streptococcal* isolates. *Infect Dis Obstet Gynecol* **2008,** *2008*, 727505.

128. Bray, R. E.; Boe, R. W.; Johnson, W. L., Transfer of ampicillin into fetus and amniotic fluid from maternal plasma in late pregnancy. *Am J Obstet Gynecol* **1966,** *96*, 938-42.

129. Fiore Mitchell, T.; Pearlman, M. D.; Chapman, R. L.; Bhatt-Mehta, V.; Faix, R. G., Maternal and transplacental pharmacokinetics of cefazolin. *Obstet Gynecol* **2001,** *98*, 1075-9.

130. Allegaert, K.; van Mieghem, T.; Verbesselt, R.; de Hoon, J.; Rayyan, M.; Devlieger, R.; Deprest, J.; Anderson, B. J., Cefazolin pharmacokinetics in maternal plasma and amniotic fluid during pregnancy. *Am J Obstet Gynecol* **2009,** *200*, 170 e1-7.

131. Popovic, J.; Grujic, Z.; Sabo, A., Influence of pregnancy on ceftriaxone, cefazolin and gentamicin pharmacokinetics in caesarean vs. non-pregnant sectioned women. *J Clin Pharm Ther* **2007,** *32*, 595-602.

132. Pacifici, G. M., Placental transfer of antibiotics administered to the mother: a review. *Int J Clin Pharmacol Ther* **2006,** *44*, 57-63.

133. Laiprasert, J.; Klein, K.; Mueller, B. A.; Pearlman, M. D., Transplacental passage of vancomycin in noninfected term pregnant women. *Obstet Gynecol* **2007,** *109*, 1105-10.

134. Philipson, A., Pharmacokinetics of antibiotics in pregnancy and labour. *Clin Pharmacokinet* **1979,** *4*, 297-309.

135. Muller, A. E.; Mouton, J. W.; Oostvogel, P. M.; Dorr, P. J.; Voskuyl, R. A.; DeJongh, J.; Steegers, E. A.; Danhof, M., Pharmacokinetics of clindamycin in pregnant women in the peripartum period. *Antimicrob Agents Chemother* **2010,** *54*, 2175-81.

136. Bolukaoto, J. Y.; Monyama, C. M.; Chukwu, M. O.; Lekala, S. M.; Nchabeleng, M.; Maloba, M. R.; Mavenyengwa, R. T.; Lebelo, S. L.; Monokoane, S. T.; Tshepuwane, C.; Moyo, S. R., Antibiotic resistance of *Streptococcus agalactiae* isolated from pregnant women in Garankuwa, South Africa. *BMC Res Notes* **2015,** *8*, 364.

137. Fitoussi, F.; Loukil, C.; Gros, I.; Clermont, O.; Mariani, P.; Bonacorsi, S.; Le Thomas, I.; Deforche, D.; Bingen, E., Mechanisms of macrolide resistance in clinical group B *streptococci* isolated in France. *Antimicrob Agents Chemother* **2001,** *45*, 1889-91.

138. Moyo, S. R.; Maeland, J. A.; Munemo, E. S., Susceptibility of Zimbabwean *Streptococcus agalactiae* (group B *Streptococcus*; GBS) isolates to four different antibiotics. *Cent Afr J Med* **2001,** *47*, 226-9.

139. Betriu, C.; Gomez, M.; Sanchez, A.; Cruceyra, A.; Romero, J.; Picazo, J. J., Antibiotic resistance and penicillin tolerance in clinical isolates of group B *streptococci*. *Antimicrob Agents Chemother* **1994,** *38*, 2183-6.

140. Park, C.; Nichols, M.; Schrag, S. J., Two cases of invasive vancomycin-resistant group B *streptococcus* infection. *N Engl J Med* **2014,** *370*, 885-6.

141. Recommended Immunization Schedule for Children and Adolescents Aged 18 Years or Younger, United States, 2018. https://www.cdc.gov/vaccines/schedules/hcp/imz/child-adolescent.html (March 5).

142. Pfizer Inc., Pfizer Awarded Grant to Evaluate Vaccine to Protect Newborns Against Group B *Streptococcus* Infection. https://www.pfizer.com/news/press-release/press-release-detail/pfizer_awarded_grant_to_evaluate_vaccine_to_protect_newborns_against_group_b_streptococcus_infection-0 (October 19, 2016).

143. Glezen, W. P.; Alpers, M., Maternal immunization. *Clin Infect Dis* **1999,** *28*, 219-24.

144. Nuccitelli, A.; Rinaudo, C. D.; Maione, D., Group B *Streptococcus* vaccine: state of the art. *Ther Adv Vaccines* **2015,** *3*, 76-90.

145. Baker, C. J.; Kasper, D. L., Correlation of maternal antibody deficiency with susceptibility to neonatal group B *streptococcal* infection. *N Engl J Med* **1976,** *294*, 753-6.

146. Baker, C. J.; Edwards, M. S., Group B *streptococcal* conjugate vaccines. *Arch Dis Child* **2003,** *88*, 375-8.

147. Baker, C. J.; Rench, M. A.; Edwards, M. S.; Carpenter, R. J.; Hays, B. M.; Kasper, D. L., Immunization of pregnant women with a polysaccharide vaccine of group B *streptococcus*. *N Engl J Med* **1988,** *319*, 1180-5.

148. Kobayashi, M.; Schrag, S. J.; Alderson, M. R.; Madhi, S. A.; Baker, C. J.; Sobanjo-Ter Meulen, A.; Kaslow, D. C.; Smith, P. G.; Moorthy, V. S.; Vekemans, J., WHO consultation on group B *Streptococcus* vaccine development: Report from a meeting held on 27-28 April 2016. *Vaccine* **2016**.

149. Heath, P. T., Status of vaccine research and development of vaccines for GBS. *Vaccine* **2016,** *34*, 2876-2879.

CHAPTER II

Human Milk Oligosaccharides Categorized by Lewis Blood Groups

Introduction

Human milk is the ideal food source for infants. Several professional bodies including the World Health Organization,[1] the American Academy of Pediatrics,[2] and the U. S. Department of Health and Human Services[3] recommend exclusive breastfeeding for the first 6 months of life, with continued integration of human milk into a mixed diet up through two years of age. The benefits associated with breastfeeding range from regulating gut microbiota to decreasing the occurrences of infections, asthma, obesity, and sudden infant death syndrome, compared to infant formula.[3-6,7,8,9,10,11] The advantages of breast feeding create a desire to understand the uniqueness of human milk relative to bovine milk, which is the basis of infant formula.

Human Milk Composition

Human milk contains both nutritional and bioactive factors that contribute to brain development, the central nervous system, and the host digestive system, while also influencing cardiovascular and metabolic health and susceptibility to infection.[12-19] In contrast to infant formula, whose content is relatively consistent, human milk has an ever-changing composition based on the individual, stage of lactation, and geographical location.[5] The earliest milk produced by mothers, known as colostrum, contains elevated levels of immunological and nutritional components and growth factors.[5, 20] Additionally, the concentration of human milk oligosaccharides (HMO) is highest in colostrum.[21-25] As an infant matures, so does the milk, and it increases levels of lactose to support the energy requirements of the growing infant.[5, 20]

On average, human milk contains 41 g/L fats, 8 g/L proteins, 70 g/L lactose, and 5-20 g/L oligosaccharides (Table 2.1).[5, 6, 26-29] One of the most striking differences between human and bovine milk types is the quantity and nature of their respective carbohydrate components. Human milk contains a higher percentage of fucosylated HMOs (50-80%), while bovine milk contains a larger portion of sialylated oligosaccharides (ca. 70%). HMOs are the third largest macromolecular component of human milk and will be the focus of this chapter. However, the other components of human milk also play an important role and will be discussed in brief below.

Table 2. 1 Comparison of macronutrients in human and bovine milk [5, 6, 27-30]

	Humans (g/L)	Bovine (g/L)
Protein	8	32
Fat	41	37
Lactose	70	48
Oligosaccharides	5-15	0.05
Percent Sialylated	10-20%	70%
Percent Fucosylated	50-80%	1%

Nutritional Components

The nutritional components of human milk remain relatively conserved and originate from enzymes in the mammary glands, circulating maternal fluids, or maternal diet.[5] Proteins, fats, and carbohydrates are macronutrients that contribute to the energy derived from milk. There is a wide array proteins found in human milk, but some of the most abundant include casein, α-lactalbumin, lactoferrin, secretory immunoglobulin A (sIgA), lysozyme, and serum albumin.[5, 31] Palmitic and oleic acids are two highly abundant fats in human milk, and lactose is the most abundant

carbohydrate.[5] Micronutrients also contribute to the nutritional aspect of human milk and can vary largely based on maternal diet. Naturally low concentrations of Vitamins K and D can be augmented by maternal diet supplementation with a multi-vitamin or infant diet supplementation with Vitamin D.[5] Other micronutrients include vitamins A, B_1, B_2, B_6, B_{12}, thiamin, riboflavin, choline, and iodine.[5, 32]

Bioactive Components

In addition to its nutritive value, human milk also contains bioactive components that play an important role in infant health and development. The immunological potential and composition of human milk depends on several factors. Nevertheless, a recent analysis of immune factors in human milk among healthy women of various ethnic, geographic, dietary, socioeconomic, and environmental circumstances revealed a core set of soluble immune factors present in all or most of the breast milk samples (n = 370).[33] These core immune factors extend across several different classes and include IgA, IgG, IgM, epidermal growth factor (EGF), Transforming growth factor β2 (TGF-β2), interleukin 7 (IL-7), IL-8, chemokine growth-regulated oncogene-α (Groα), and macrophage inflammatory protein β (MIP1β).

Several growth factors are found in human milk and have diverse effects on multiple biological systems. For instance, EGF can withstand the harsh environment of the digestive tract and stimulate the growth of cells that are important for intestinal maturation and repair.[34-36] The growth and development of the enteric nervous system relies on brain-derived and glial cell-line derived neurotrophic factors.[37-40] These neurotrophic factors can increase peristalsis and neuron survival and outgrowth. Tissue growth is stimulated in part by the insulin-like growth factor (IGF) superfamily.[5] Vascular endothelial growth factor (VEGF) regulates angiogenesis, an important

part of the vascular system.[5, 41] Erythropoietin (Epo) is one of the hormones found in human milk and contributes to the prevention of anemia through increasing red blood cells.[42, 43] Epo has also been shown to be important in intestinal development and protection against necrotizing enterocolitis.[44, 45] Several hormones linked to metabolic regulation are found in human milk: adiponectin, leptin, resistin, and grehlin.[46-49] These influence infant metabolism, energy conversion, and appetite control. Other growth-regulating hormones, such as calcitonin and somatostatin, are also found in human milk, but their specific role has yet to be fully elucidated.[5] Just as calcitonin increases calcium concentration in the gut and kidney, it is likely that it has a similar effect in the mammary gland to increase calcium levels transferred to the neonate.[50]

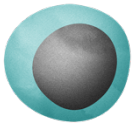
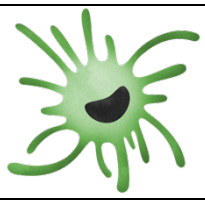
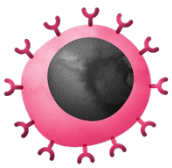
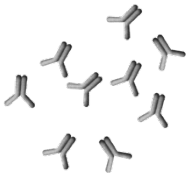
Human milk is rich with innate immune factors that provide protection against infection and inflammation in infants (Table 2.2). For example, a collection of living cells such as macrophages, T cells, stems cells, and lymphocytes are found in human milk. These cells play an active role in conferring protection and initiating the programming for the neonate's immune system.[5, 51] Nearly 80% of these cells are macrophages and participate in phagocytosis of various pathogens or differentiate into dendritic cells that facilitate communication between the innate and adaptive immune systems.[5, 52] Human milk stem cells help establish a microchimeric state in the infant and likely participate in tissue and immune cell regeneration.[51, 53, 54]

Human milk also contains several cytokines and chemokines, important classes of immune factors, that participate in cell signaling and communication between cells (Table 2.2). Many cytokines act in concert with other immune regulators to produce a domino effect that contributes to the overarching development and responses of the immune system.[56] Cytokines found in human milk can be categorized into two broad groups: those that reduce inflammation or those that

enhance inflammation and defend against infection.[5] TGF-β2 is the most abundant cytokine in human milk and is one of three isoforms of the multifunctional TGF-β family.[5, 57, 58] Considering endogenous TGF-β synthesis is lacking during neonatal development, TGF-β2 is an important immunomodulatory factor of breast milk because it induces oral tolerance in infants that leads to prevention of food allergies and to regulation of immune responses in the intestinal tract.[33, 59-61] Some of the regulatory cytokine found in human milk include granulocyte-colony stimulating factor (G-CSF), IL-10, and IL-7. These cytokines are responsible for aspects of intestinal development including cell proliferation and differentiation at the intestinal surface.[62, 63] Specific roles of many pro-inflammatory cytokines in human milk—tumor necrosis factor α (TNF-α), IL-6, IL-8, and interteron γ (IFNγ)—have yet to be identified. However, IL-8 is involved in the chemotaxis of leukocytes from maternal cells to human milk.[64] The chemokine Groα, also known as C-X-C motif ligand 1 (CXCL1), plays an important role in neuroprotection and angeogenesis.[65, 66] Still, other chemokines such as MIP1β, also known as C-C motif ligand 4 (CCL4), MIP1α, and RANTES (regulated on activation, normal T cell expressed and secreted) play a role in suppressing HIV.[67]

As infants develop their immune system, several immunoglobulins, a type of glycoprotein found in human milk, provide protection against pathogen invasion. sIgA, the most predominant immunoglobulin, influences the potential development of allergic diseases and the immune response to dietary antigens.[68, 69] IgA, IgM, and IgG contribute to protection against pathogen colonization and invasion.[33, 70-72] In addition to their effect on other pathogens, immunoglobulin antibodies have been shown to protect the neonate from GBS infection by interfering with carbohydrate-mediated attachment to epithelial cells or serving as antibodies of GBS CPS.[20, 73-77]

Table 2. 2 Immune Cells found in Human Milk[55]

Cell Type	Illustration	Function
Stem Cell		Tissue and immune cell regeneration
Neutrophils		Phagocytosis Enzyme release
Macrophages		Phagocytosis Inflammation Phenotypic plasticity Cytokine and chemokine Secretion
Dendritic Cells		Phagocytosis Produce cytokines Display antigens to T-cells
Eosinophils		Allergy response Cytotoxicity Produce growth factors and cytokines
T-cell		Recognize antigens Stimulate immune cells Cytotoxicity
Antibodies		Recognize and bind antigens for clearance of invaders
Cytokine and chemokines		Cell signaling and communication

Other glycoproteins in human milk also contribute to the function of an infant's innate immune system. Lactoferrin is an iron-binding glycoprotein that has antimicrobial activity against

several viral, fungal, and bacterial pathogens.[78-80] Lactoferrin also plays a role in host defense by binding to bacterial membranes, inhibiting TNF- α and IL-1β, and stimulating the activity and development of lymphocytes.[51, 81] Lactadhedrin is a glycoprotein that confers protection against rotaviral infections and aids in recovery from intestinal inflammations.[5, 82] Lactadhedrin also triggers signaling cascades involved in phagocytosis by acting as a bridge for macrophage recognition of lactadhedrin bound to the phosphatidylserine on apoptotic cells.[83, 84] Other glycoproteins that protect against pathogen invasion by acting as decoy receptors include Mucin 1 (MUC1), MUC4, and several gangliosides (GM1, GM3, and GD3).[51, 85]

In addition to nutritional and immunological components, human milk also contains a multitude of bacterial species. Both culture-dependent and genomic-based approaches have been used to characterize the milk microbiome. Some of the bacterial species present in human milk include *Staphyloccocus*, *Streptoccocus*, *Propionibacterium*, *Pseudomonas*, and *Lactobacillus*.[51, 86-89] There are approximately 400 species of bacteria in human milk at any given time.[88, 90] Many of these bacteria are commensal or symbiotic and provide infants with the infrastructure to develop a healthy gut micriobiome.[91-93] Human milk is among the earliest vehicles for intestinal bacterial colonization but can also be a source of pathogenic bacteria that leads to newborn diseases.[14, 73, 94-96] Similar to many of the immune factors found in human milk, the concentration and types of bacteria are dependent on several factors such as geographical region, gestational age, genetics, mode of delivery, and maternal nutrition.[97, 98]

Carbohydrate Components

Human milk contains a substantial carbohydrate portion, of which lactose is the main carbohydrate constituent. Lactose serves as an energy source after digestion into its

monosaccharide components (glucose and galactose). Most cells utilize glucose for energy, and galactose can also be used for energy after it is converted to glucose in the liver.[99]

In addition to lactose, human milk contains an abundant and structurally diverse set of carbohydrates known as human milk oligosaccharides (HMOs). In contrast to lactose, the oligosaccharides present in human milk are not digestible by infants.[25, 100, 101] Instead, HMOs travel to the infant gut where they serve as prebiotics and help regulate the development of the infant gut microbiome by stimulating the growth of beneficial microoranisms such as bifidobacteria.[100-103] In turn, the composition of the microbiome can influence an infant's health.[104] Certain infant gut-associated bifidobacteria have genes and enzymes dedicated to HMO utilization, allowing them to thrive on HMOs as a carbon source.[105] HMOs can also serve as antiadhesive antimicrobial agents and lower the risk of infections by functioning as decoy receptors for various pathogens.[102, 106-110] Before host invasion, many pathogens first adhere to epithelial surfaces through lectin-glycan interactions. Some HMOs resemble the glycans on the surface of epithelial cells, and pathogens will bind to these HMOs rather than the host cells. Additionally, sialylated HMOs are thought to be important in brain development as sialic acid is an essential component of brain gangliosides and neural cell adhesion molecules.[102, 111, 112]

While the functions of many individual HMOs remain unclear, a few structure-activity relationships have been described. For instance, 2'-fucosyllactose (2'-FL), one of the most prevalent HMOs, exhibits anti-inflammatory activity by modulating signaling cascades that result from *E. coli* infections.[113] Specifically, 2'-FL inhibits membrane-bound CD14 expression, an important component of a complex that activates signaling pathways for the production of inflammatory mediators such as IL-8.[113] Another HMO, disialyllacto-N-tetraose (DSLNT) has an

inhibitory effect on necrotizing entercolitis (NEC) in a neonatal rat model.[114] While the exact mechanism of NEC prevention is unknown, it is likely to occur through signaling cascades mediated by the binding of DSLNT to siglec receptors, disrupting the interaction between siglecs and toll-like receptors to activate an immune response.[114-116]

Discovery of HMOs

A distinction in the composition of human and bovine milk was first noted in 1888. At this time, Eschbach observed that human milk contained a "more heterogeneous form of lactose" while bovine milk contained a "more homogenous form of lactose."[117] Researchers concluded that lactose was identical in both milks, but noted an additional carbohydrate fraction that had not yet been specified. This fraction remained unnamed until nearly 40 years later when it was termed "gynolactose" by two French chemists, Michel Polonowski and Albert Lespagnol.[102, 117-119] They rudimentarily described gynolactose as containing nitrogen, hexosamines, and other carbohydrates. Soon after, Polonowski and Montreuil used 2-dimensional paper chromatography to identify 2'-FL and 3-fucosyllactose (3-FL) in the previously uncharacterized human milk fraction.[117, 120] The initial identification of these components prompted the desire to characterize "gynolactose" more fully by assigning structural aspects to its components. Research by both Montreuil and Kuhn led to a clear description of several HMOs, including the previously described 2'-FL and 3-FL as well as difucosyllactose (DF-L), lacto-*N*-tetraose (LNT), lacto-*N*-fucopentaose I (LNF-I), LNF-II, and lacto-*N*-difucohexaose I (LNDF-I) among others.[117, 121-130] Some of the oligosaccharides in human milk showed similar activities to blood group determinants because of their structural resemblance (Figure 2.1). Specifically, 2'-FL and LNF-I simulated the activity of H determinant, LNDF-I the activity of Le^b determinant, and LNF-II the activity of Le^a

determinant.[128, 131-134] The correlation between HMOs and blood groups led to a surge in the elucidation of new HMO structures that continues today.[131, 135-142]

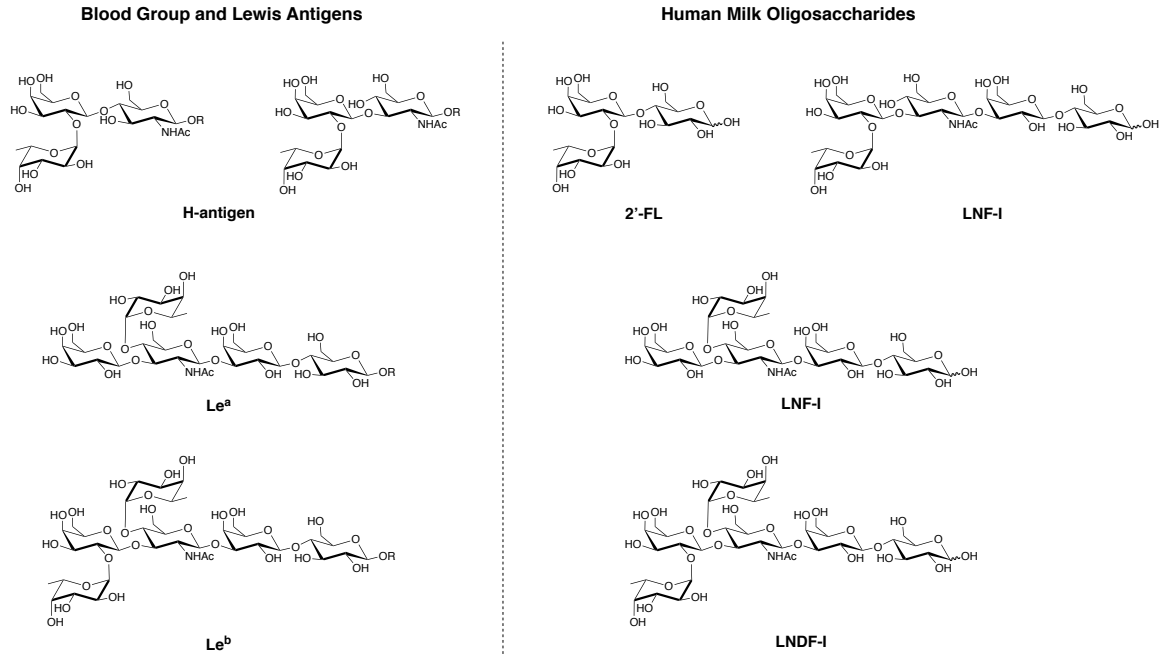


Figure 2. 1 Comparison of Blood Group Antigens and HMOs. The blood group H-antigen and Lewis structures found in glycan-containing molecules compared to HMOs that have similar or identical carbohydrate structures. Abbreviations: 2′-FL, 2′-fucosyllactose; LNF-I, lacto-*N*-fucopentaose I; LNDF-I, Lacto-*N*-difucohexaose I.

Escherich, an Austrian pediatrician and microbiologist, in the late 19th century made a connection between intestinal bacteria and physiology of an infant's digestion. This initial observation led to the appreciation of the differences in microbiota of breast-fed infants compared to those who were bottle-fed and furthered the notion that human milk has unique components that contribute to infant health and development. The chemical nature of these components remained unknown until a connection was made between the work on "gynolactose" and the growth-promoting factor for bacteria, establishing oligosaccharides as a "bifidus factor." Since then,

structural elucidation and functional studies of HMOs have continued to reveal a much greater influence of HMOs on infant health beyond serving as a "bifidus factor."

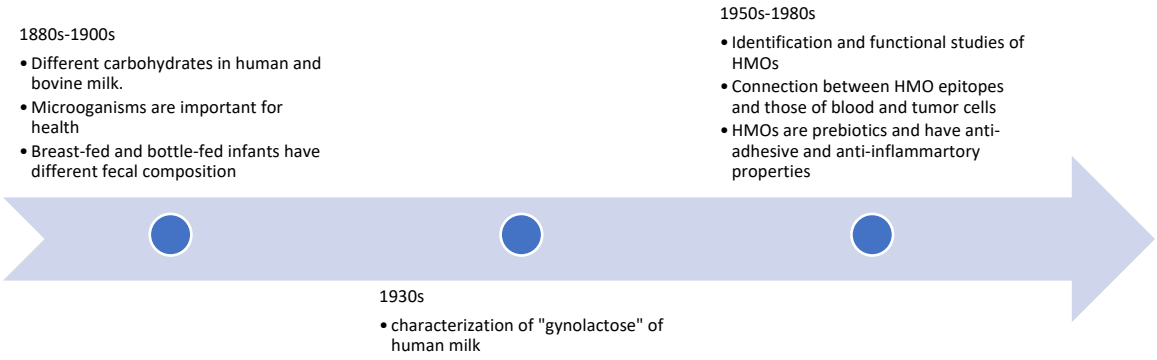


Figure 2. 2 Timeline of HMO research in the 20th century. It was pioneered by pediatricians and microbiologists who studied the benefits of human milk for the breast-fed infant and by chemists who worked to identify the unique carbohydrate portion of human milk. This led to the characterization of the "gynolactose" portion of human milk and subsequent identification of over 100 unique HMO structures along with functional studies of HMOs.[26]

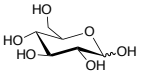
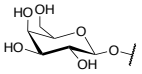
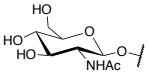
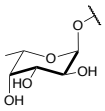
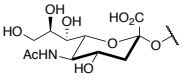
HMO Structure

HMO composition and concentration vary among women and over the course of lactation, but five monosaccharides are incorporated into HMO structures: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and N-acetylneuraminic acid (Neu5Ac, also known by its common name, sialic acid, Sia) (Table 2.3). [26, 102, 143] There are estimates that over 200 unique structures exist.

HMOs contain lactose (Galβ1-4Glc) at the reducing end and can be elongated with lacto-N-biose (Galβ1-3GlcNAc) to form type 1 chains or N-acetyllactosamine (Galβ1-4GlcNAc) to form type 2 chains (Figure 2.3A). HMOs range from 3 to 32 monosaccharide units.[145, 146] Acidic HMOs have core structures that are decorated with sialic acid residues in an α-2,3 or α-2,6 linkage

to terminal galactose or in an α-2,6 linkage to GlcNAc. Fucose residues decorate core structures in α-1,2, α-1,3, or α-1,4 linkages in neutral HMOs. Sialylated HMOs have a lower abundance (10-20%) relative to fucosylated HMOs (50-80%).[6, 27, 28, 102, 131, 141, 142, 147-151] This is in contrast to the relative abundances of sialylated and fucosylated oligosaccharides in bovine milk, which is ca. 70% and 1% respectively.[102, 152, 153] The abundance of fucosylated HMOs in human milk is suited for the protective role many of these oligosaccharides play.

Table 2. 3 Major Monosaccharide Building Blocks of HMOs[144]

Monosaccharide	Abbreviation	Symbol	Structure	Linkage
Glucose	Glc			None (at the reducing end)
Galactose	Gal			β
N-Acetylglucosamine	GlcNAc			β
Fucose	Fuc			α
N-Acetylneuraminic acid (sialic acid)	Neu5Ac (Sia)			α

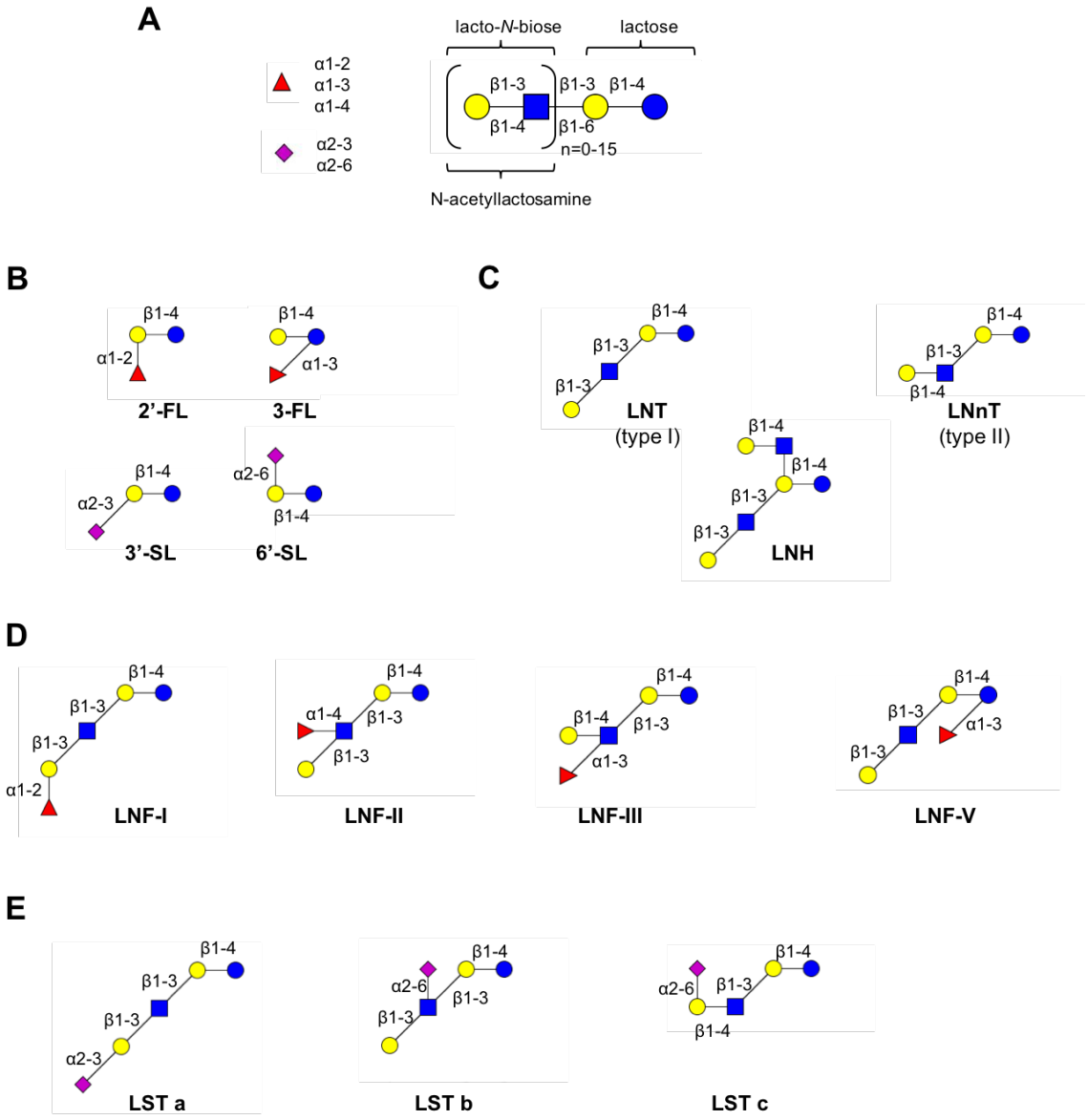


Figure 2. 3 Structure of Several HMOs. A) Basic structure of HMOs. HMOs have lactose at the reducing end and can be elongated by lacto-N-biose or N-acetyllactosamine and further decorated with fucose or sialic acid residues. B) Lactose can be fucosylated or sialylated by different linkages. C) Lactose can be elongated by lacto-N-biose (type I) or N-acetyllactosamine (type II). Elongated chains can be D) fucosylated or E) sialylated. Abbreviations: 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNH, lacto-N-hexaose; LNF-I, II, II, V, lacto-*N*-fucopentaose I, II, III, V; LST a, b, c, sialyl-lacto-N-tetraoses a–c.[26]

Pioneering work by Victor Ginsburg demonstrated that HMO structural features are

determined by gene-regulated expression of specific glycosyltransferases. The expression of these transferases, and consequently oligosaccharide structure and composition, are influenced by the mother's Lewis blood group and secretor status.[154, 155] Lewis blood groups, which are determined by the presence or absence of secretor and Lewis genes, dictate the expression of three different types of fucosyltransferases. Secretor mothers possess an active *Se* gene locus encoding for the fucosyltransferase II (FUT2). FUT2 transfers fucose in an α-1,2 linkage to a terminal galactose, resulting in milk that is rich in α-1,2 fucosylated HMOs. Nonsecretors lack an active *Se* locus and do not produce HMOs with this glycosidic linkage. Lewis positive mothers have an active *Le* gene locus encoding for the α-1,3 and α-1,4 fucosyltransferase FUT3 which installs fucose in an α-1,4 linkage to N-acetylglucosamine. Several fucosyltransferases (FucT) are responsible for installing α-1,3 fucosyl linkages to either N-acteylglucosamine or glucose. Because Lewis negative mothers do not have an active *Le* locus, their milk lacks α-1,4 fucosylated HMOs.

The distribution of Lewis blood groups across a given population varies based on geographical and ethnic factors. Recorded distributions for American populations are shown in Table 2.4. The largest percentage of the population (55-72%) are individuals belonging to the Lewis (a-b+) blood group, which express α-1,2FucT, α-1,3FucT, and α-1,4FucT. Lewis (a+b-) individuals account for 20-23% of the population and express α-1,3 FucT and α-1,4 FucT. Lewis (a-b-) express α-1,2 FucT and α-1,3FucT and account for 5-10% of the population. Lewis (a-b-c+d-) is a very rare Lewis blood group, where an individual does not express any fucosyltransferases, and makes up less than 1% of the population.[156]

Table 2. 4 Lewis blood group determinants. The expressed enzymes and genotypes corresponding to each Lewis blood group are listed along with their prevalence as a percentage of the general population.[133, 134]

Lewis Blood Group	Expressed Enzymes	Genotype		Percent of Population
		Secretor	Lewis	
Lewis (a-b+)	α1-2 FucT α1-3 FucT α1-4 FucT	Se/-	Le/-	55-72
Lewis (a+b-)	α1-3 FucT α1-4 FucT	se/se	Le/-	20-23
Lewis (a-b-)	α1-2 FucT α1-3 FucT	Se/-	le/le	5-22
Lewis (a-b-c+d-)	-	se/se	le/le	≤1

Biosynthesis of HMOs

The biosynthesis of HMOs begins with the functionalization of a lactose core. Lactose synthesis is well studied and known to occur in the Golgi of the mammary glands by the action of the lactose synthase complex that consists of two enzymes: α-lactalbumin and β1-4 galactosyltransferase (β1-4GalT1) (Figure 2.4).[102, 157] In the absence of α-lactalbumin, β1-4GalT1 transfers UDP-Gal to GlcNAc.[102] However, as part of the lactose synthase complex, β1-4GalT1 transfers UDP-Gal to glucose to yield lactose.[158, 159]

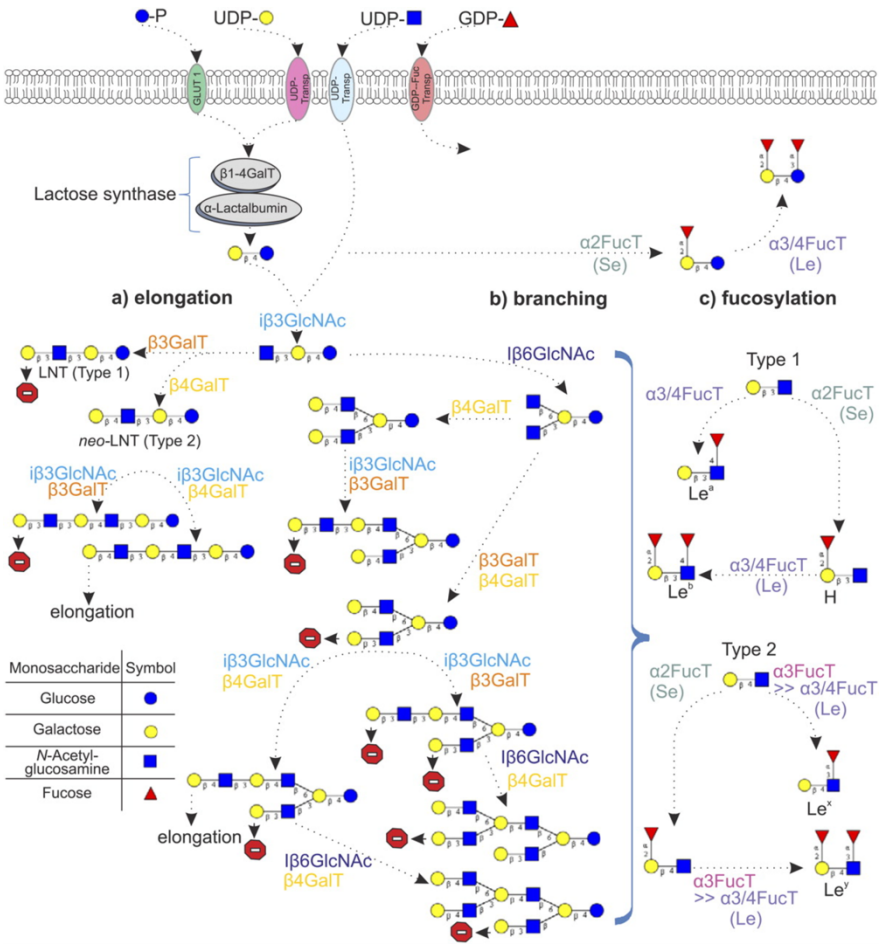


Figure 2. 4 Biosynthesis of neutral complex human milk oligosaccharides (HMO). The assumed biosynthetic pathway starts from the activated monosaccharides and includes the most important enzymes only [N-acetylglucosaminyltransferases (GlcNAcT)]: iβ3GlcNAcT attaches N-acetylglucosamine (GlcNAc) in the β1–3 position to terminal galactose (Gal), Iβ6GlcNAcT attaches GlcNAc in β1–6 position to terminal Gal. Galactosyltransferases (GalT): β3GalT attaches Gal in the β1–3 position to GlcNAc and β4GalT attaches Gal in the β1–4 position to GlcNAc. Fucosyltransferases (FucT): α2FucT attaches fucose (Fuc) in the α1–2 position to terminal Gal, secretor (Se) enzyme, α3FucT attaches Fuc in the α1–3 position to GlcNAc, α3/4FucT attaches Fuc in the α1–3/4 position to GlcNAc and in the α1–3 position to Glc of the lactose core, Lewis (Le) enzyme. The no entry signs mean that no further elongation takes place. Fucosylation is indicated exemplarily for terminal type 1 and type 2 chains. Glycan structures are depicted according to the recommendations of the Consortium of Functional Glycomics using the GlycoWorkbench software tool. (Reproduced with permission from: Human Milk Oligosaccharides and Lewis Blood Group: Individual High-Throughput Sample Profiling to Enhance Conclusions from Functional Studies. *Adv Nutr*. **2012**; 3 (3):440S-449S. doi:10.3945/an.111.001446. Adv Nutr | © 2012 American Society for Nutrition)

Elongation of the lactose core is less understood because many of the specific glycosyltransferases required for the biosynthesis of HMOs have not been identified.[144] Still, significant progress has been made in understanding the fucosylation patterns and associated

fucosyltransferases that are dependent upon blood group characteristics.[102] It is likely that HMOs are elongated by extension of the lactose core via addition of monosaccharides by alternating actions of requisite glycosyltransferases (Figure 2.4).[102, 160] For example, elongation to form the tetrasaccharide LNT would first require the action of an N-acteylglucosaminyltransferase (β3GalNAcT) to install GlcNAc on lactose, followed by a galactosyltransferase to install Gal on GlcNAc (Figure 2.4). Functionalization of the lactose core or elongated chains by other monosaccharides is accomplished by the action of a glycosyltransferase specific for each monosaccharide and each type of linkage. To date, several glycosyltransferases have been purified from human milk: β1-4GalT1, α1-3FucT, α-1,4FucT, and α-1,3/4FucT.[161-166]

Identification and Characterization of HMOs

The complex and diverse branching of HMO structures and the lack of amplification techniques has made the identification and characterization of HMOs a challenging undertaking.[167] Initial characterizations of HMOs was carried out using classical analytical methods.[117] However, advanced techniques, including spectroscopic, chromatographic, and electromigration methods, have led to improved characterizations of mixtures for individual identification and quantification of HMOs.[117, 167] Separation techniques coupled with mass spectrometric analysis are extensively used for both the qualitative and quantitative characterization of HMOs.[167]

Liquid chromatography is the most common methods for HMO separation and characterization, and includes reversed-phase (RPC), high-pH anion-exchange (HPAEC), porous graphite carbon (PGC), normal-phase (NPC), and hydrophilic interaction (HILIC) chromatography.[167] RPC can be used to study neutral oligosaccharides but requires pre-column derivatization to increase separation by this high-performance liquid chromatography (HPLC)

technique.[168-170] HPAEC has been used to analyze both acidic and neutral HMOs.[171, 172] While HPAEC does not require any derivatization, acidic and neutral HMOs must be separated prior to analysis. PGC has been used for the characterization of isomeric species after reduction to the respective alditols.[167, 173] This method has also been used for the analysis of both neutral and acidic HMOs.[174, 175] In NPC and HILIC techniques, a polar stationary phase and an apolar mobile phase lead to the separation of HMOs and allow for simultaneous determination of both neutral and acidic portions.[24, 167, 176, 177] Fluorescent derivatization has been used to improve the sensitivity of these methods.[176, 178, 179]

Gas chromatography (GC) is less widely employed to separate and identify HMOs.[167] Nevertheless, after acidic hydrolysis of HMOs, GC has been employed in some of the earliest studies to identify the monosaccharide constituents of HMOs.[135, 136] More recent uses of GC for the identification of HMOs take advantage of the improved volatility of trimethylsilyl oxime derivatives of oligosaccharides.[180]

Other techniques to analyze HMO samples include electromigration methods. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are employed for the separation of charged species, making these methods especially useful for studying sialylated HMOs.[149, 167, 181, 182] These methods can also be used to study neutral HMOs after initial derivatization to generate an anionic species.[183, 184]

In addition to identifying individual HMOs in a complex mixture, some of these techniques can also be used to characterize human milk samples based on the presence or absence of specific oligosaccharides. As mentioned previously, the secretor and Lewis status of the mother is directly linked to the types and quantities of HMOs present in milk.[26, 106, 132, 185] Since fucosylated glycans

are abundant in human milk and can be used to distinguish between Lewis blood groups, identification of fucosylation patterns have been used to characterize human milk samples according to the Lewis blood group of the donor. Specifically, recent reports have demonstrated the use of mass spectrometry and NMR for this purpose.[186-188] NMR analysis focuses on the change in chemical shift between α-1,2, α-1,3, and α-1,4 fucosylated oligosaccharides (Figure 2.5).[189] Kunz and coworkers developed a high throughput mass fingerprinting technique that uses matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF tandem mass spectrometry (MS/MS) to identify major fucose-containing oligosaccharides and their fucosyl linkage types and subsequently assign the corresponding Lewis blood group (Figure 2.6).[186]

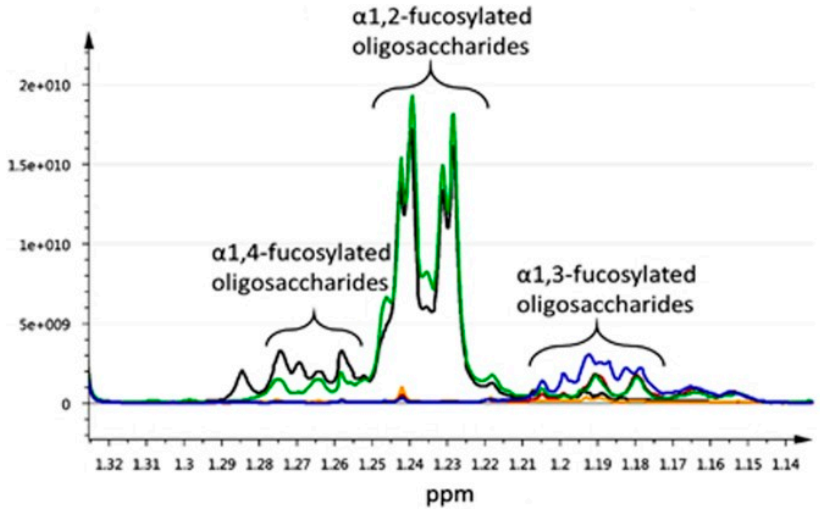


Figure 2. 5 Comparison of spectra of mothers producing different profiles of HMOs. Spectra from mothers in blue, orange and red are non-Secretors, as these spectra do not contain signals corresponding to 2′-FL between δ 1.22 and 1.25, while the mothers in green and black are classified as Secretors. (Reproduced with permission from Andreas, N. J., Al-Khalidi, A., Jaiteh, M., Clarke, E., Hyde, M. J., Modi, N., Holmes, E., Kampmann, B. and Mehring Le Doare, K. (2016), Role of human milk oligosaccharides in Group B Streptococcus colonisation. Clin Trans Immunol, 5: n/a, e99. doi:10.1038/cti.2016.43))

Results

Assigning Lewis Blood Groups to Human Milk Samples

Based on the premise that human milk contains a variety of anti-infective agents, we hypothesized these molecules, specifically HMOs, can modulate Group B strep physiology. In order to test this hypothesis, isolation and characterization of HMOs from human milk samples was necessary.

HMOs isolated from the milk of 19 different donors were kept separate, and Kunz's methodology was used to assign Lewis blood groups to each sample. These assignments were then used to evaluate the hypothesis that Lewis blood groups are correlated to anti-bacterial activity of HMOs on GBS (Chapter 3).

MALDI-TOF and subsequent MS/MS fragmentations of parent peaks *m/z* 657 and 1022 was performed. These two peaks represent difucosylated oligosaccharides whose fragmentation patterns are correlated with the fucosyl linkages used to assign Lewis blood groups. Fragmentation of *m/z* 657 that results in a fragment peak at m/z 511 is associated with Le(a-b+) and Le(a-b-) blood groups, which both express the enzyme to form α-1,2 fucosyl linkages (FUT2) (Figure 2.6). The absence of a fragmentation peak at m/z 511 corresponds to Le(a+b-), which only has α-1,3 and α-1,4 fucosyl linkages. To distinguish between Le(a-b+) and Le(a-b-) blood groups, the relative abundance of the fragment ions of m/z 1022 can be used. Le(a-b+) has a lower m/z 730 relative to m/z 876, whereas Le(a-b-) has a similar or higher abundance of m/z 730 compared to m/z 876.[186]

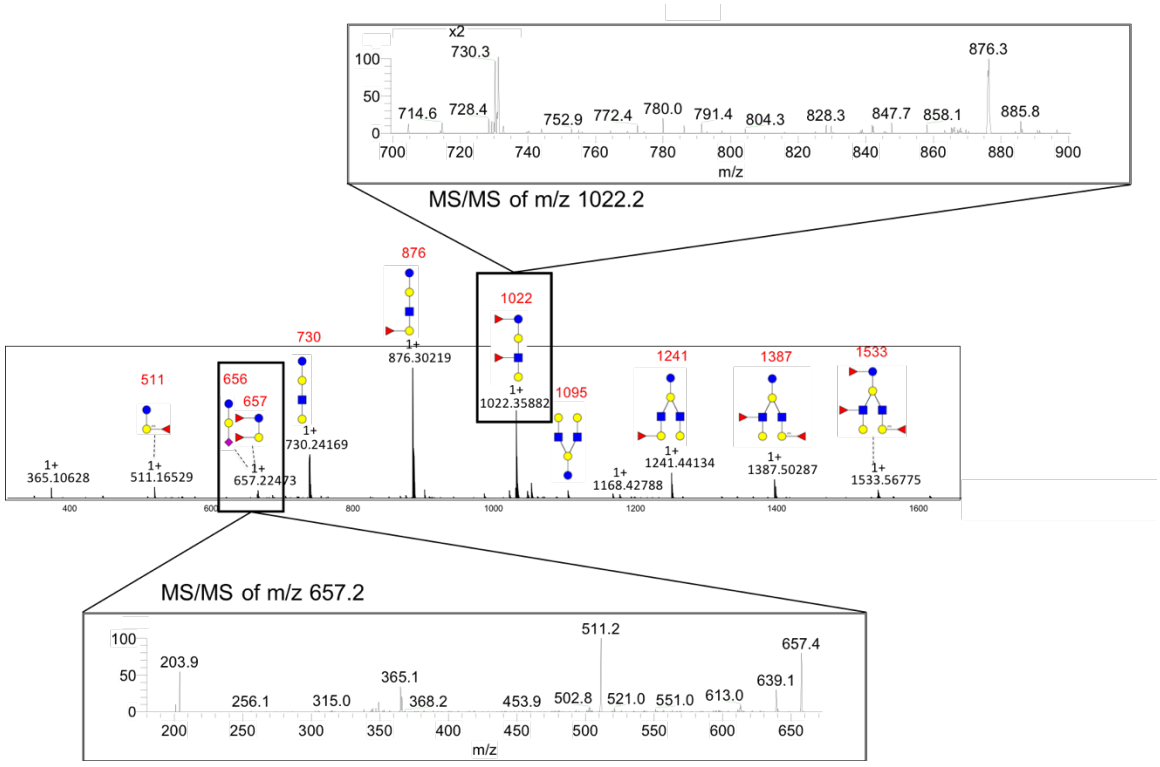


Figure 2. 6 Representative MALDI-TOF MS HMO profile and corresponding MALDI-TOF MS/MS of m/z 657 and m/z 1022 for HMOs from an individual donor. Signals obtained at m/z 657 represent an isotopic signal of sialyllactose (m/z 656) or the summed signals of difucosyllactose (m/z 657) plus the isotopic signal of sialyllactose (m/z 656). Signals obtained at m/z 1022 represent a signal of difucosylated lactose. The fragmentation of these two m/z can be used to determine the corresponding Lewis blood of group of the donor. Fragment ions of interest are m/z 511 (from m/z 657) and the ratios of m/z 730 and 876 (from m/z 1022).

Analysis of MS/MS of m/z 657 revealed donors 0, 5, 7, 8, 14, 16, 19, 20, 24, 32, 34, 37, 38, and 42 to have a fragmentation peak of m/z 511, which is associated with Le(a-b+) and Le(a-b-). Donors 17, 18, 29, 31, and 43 were missing this characteristic fragment ion and designated as Le(a+b-) (Table 2.5). Distinguishing between Le(a-b+) and Le(a-b-) required analysis of MS/MS of m/z 1022. Donors 0, 5, 7, 8, 14, 19, 20, 24, 32, 34, 37, and 42 were identified as Le(a-b+), and donors 16 and 38 were identified as Le(a-b-) (Table 2.5). The distribution of Lewis blood groups for the mothers in this study tracks well with distributions reported previously for larger populations (Table 2.6).[99, 143, 154]

Table 2. 5 Human milk donor designation and assigned Lewis blood groups

Donor	Lewis Blood Group
0	a-b+
5	a-b+
7	a-b+
8	a-b+
14	a-b+
16	a-b-
17	a+b-
18	a+b-
19	a-b+
20	a-b+
24	a-b+
29	a+b-
31	a+b-
32	a-b+
34	a-b+
37	a-b+
38	a-b-
42	a-b+
43	a+b-

Table 2. 6 Comparison of assigned Lewis blood group distribution to expected distribution

Lewis Blood Group	Out of 19 Donor	Distribution of Donors	Expected Distribution of Donors
Lewis (a-b+)	12	63%	55-72%
Lewis (a+b-)	5	26%	20-23%
Lewis (a-b-)	2	11%	5-22%

Conclusion

The oligosaccharides in human milk are distinct from that of other mammals and are one of many bioactive components found in human milk. While several studies have shown the ability of HMOs to confer health and developmental benefits to the infant during the postnatal period, many of them have reported activity without describing specific structure-activity relationships However, some of these studies revealed disruption of pathogen adhesion and infection caused by HMOs. We hypothesized that the HMO composition of milk samples could be characterized by mass spectrometry and this information used to draw correlations between any activity and HMO composition. Specifically, we were interested in the effects of HMOs on the growth and biofilm formation of GBS. While the Bode and Le Doare research groups have separately shown the ability of HMOs to modulate the colonization and growth of GBS, our goal was to further investigate the relationship between HMO composition associated with Lewis blood group status and the extent of activity against GBS.

Methods

Purification of HMOs

Human milk was obtained from nineteen healthy, lactating women between 3-90 days postnatal and stored at -20°C. The de-identified milk was provided by Dr. J. H. Weitkamp from the Vanderbilt Department of Pediatrics under a collection protocol approved by the Vanderbilt University Institutional Review Board (IRB#100897). Milk samples and the respective components from subsequent purification steps were kept separate. The lipid components were removed by skimming after centrifugation. Proteins were precipitated by addition of ethanol at 4

°C and subsequent centrifugation. The HMO-containing supernatant was concentrated *in vacuo*, purified by P-2 Gel (H_2O eluent), and the oligosaccharides were dried by lyophilization.

MS and MS/MS Analysis of HMO Samples

Dried HMO samples were reconstituted in water to approximately 1 mg/mL. The HMO solutions were deposited on a matrix-assisted laser desorption/ionization (MALDI) target plate as follows: 1 μL HMO solution was spotted followed by 0.2 μL 10 mM NaCl and 1 μL DHB matrix (60 mg/mL in 50% methanol). The spots were allowed to air dry, then analyzed in positive ion mode on a 9.4 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS) (Bruker Solarix). Mass spectra were acquired from *m/z* 300-2500. Sodium ion adducts of HMO's were detected with a mass accuracy of >2 ppm.

MS/MS analysis was performed for selected ions with a linear ion trap mass spectrometer equipped with a MALDI source (LTQ XL, Thermo Scientific). Selected sodium ion adducts of interest were isolated with a 1 amu window and fragmented via CID using a collision energy of 35 eV.

References

1. *Exclusive breastfeeding for six months best for babies everywhere*; World Health Organization: 15 January, 2011.

2. Eidelman, A. I.; Schanler, R. J.; Johnston, M.; Landers, S.; Noble, L.; Szucs, K.; Viehmann, L.; Feldman-Winter, L.; Lawrence, R.; Kim, S.; Onyema, N.; Breastfeeding, S., Breastfeeding and the Use of Human Milk. *Pediatrics* **2012,** *129* (3), E827-E841.

3. Mass, S., Supporting breastfeeding in the United States: the Surgeon General's call to action. *Current Opinion in Obstetrics & Gynecology* **2011,** *23* (6), 460-464.

4. Guaraldi, F.; Salvatori, G., Effect of breast and formula feeding on gut microbiota shaping in newborns. *Front Cell Infect Microbiol* **2012,** *2*, 94.

5. Ballard, O.; Morrow, A. L., Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am* **2013,** *60* (1), 49-74.

6. Coppa, G. V.; Pierani, P.; Zampini, L.; Carloni, I.; Carlucci, A.; Gabrielli, O., Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr Suppl* **1999,** *88* (430), 89-94.

7. Gartner, L.; Morton, J.; Lawrence, R.; Naylor, A.; O'Hare, D.; Schanler, R.; Eidelman, A., Breastfeeding and the use of human milk. *Pediatrics* **2005,** *115* (2), 496-506.

8. James, D.; Lessen, R., Position of the American Dietetic Association: Promoting and Supporting Breastfeeding. *J Am Diet Assoc* **2009,** *109* (11), 1926-1942.

9. Bartick, M., Breastfeeding and Health: A Review of the Evidence. *J Women Politics Policy* **2013,** *34* (4), 317-329.

10. Lamberti, L.; Walker, C.; Noiman, A.; Victora, C.; Black, R., Breastfeeding and the risk for diarrhea morbidity and mortality. *Bmc Public Health* **2011,** *11*.

11. Ip, S.; Chung, M.; Raman, G.; Chew, P.; Magula, N.; DeVine, D.; Trikalinos, T.; Lau, J., Breastfeeding and maternal and infant health outcomes in developed countries. *Evid Rep Technol Assess (Full Rep)* **2007,** (153), 1-186.

12. de Halleux, V.; Rigo, J., Variability in human milk composition: benefit of individualized fortification in very-low-birth-weight infants. *Am J Clin Nutr* **2013,** *98* (2), 529S-35S.

13. Donovan, S. M., Role of human milk components in gastrointestinal development: current knowledge and future needs. *J Pediatr* **2006,** *149* (5), S49-S61.

14. Donovan, S. M.; Wang, M.; Li, M.; Friedberg, I.; Schwartz, S. L.; Chapkin, R. S., Host-microbe interactions in the neonatal intestine: role of human milk oligosaccharides. *Adv Nutr* **2012,** *3* (3), 450S-5S.

15. Isaacs, E. B.; Gadian, D. G.; Sabatini, S.; Chong, W. K.; Quinn, B. T.; Fischl, B. R.; Lucas, A., The effect of early human diet on caudate volumes and IQ. *Pediatr Res* **2008,** *63* (3), 308-14.

16. Lucas, A.; Morley, R.; Cole, T. J.; Lister, G.; Leeson-Payne, C., Breast milk and subsequent intelligence quotient in children born preterm. *Lancet* **1992,** *339* (8788), 261-4.

17. Singhal, A., Early nutrition and long-term cardiovascular health. *Nutr Rev* **2006,** *64* (5 Pt 2), S44-9; discussion S72-91.

18. Ronnestad, A.; Abrahamsen, T. G.; Medbo, S.; Reigstad, H.; Lossius, K.; Kaaresen, P. I.; Egeland, T.; Engelund, I. E.; Irgens, L. M.; Markestad, T., Late-onset septicemia in a Norwegian national cohort of extremely premature infants receiving very early full human milk feeding. *Pediatrics* **2005,** *115* (3), e269-76.

19. Sisk, P. M.; Lovelady, C. A.; Dillard, R. G.; Gruber, K. J.; O'Shea, T. M., Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. *J Perinatol* **2007,** *27* (7), 428-33.

20. Andreas, N. J.; Kampmann, B.; Mehring Le-Doare, K., Human breast milk: A review on its composition and bioactivity. *Early Hum Dev* **2015,** *91* (11), 629-35.

21. Asakuma, S.; Urashima, T.; Akahori, M.; Obayashi, H.; Nakamura, T.; Kimura, K.; Watanabe, Y.; Arai, I.; Sanai, Y., Variation of major neutral oligosaccharides levels in human colostrum. *Eur J Clin Nutr* **2008,** *62* (4), 488-94.

22. Urashima, T.; Asakuma, S.; Leo, F.; Fukuda, K.; Messer, M.; Oftedal, O. T., The Predominance of Type I Oligosaccharides Is a Feature Specific to Human Breast Milk. *Adv Nutr* **2012,** *3* (3), 473s-482s.

23. Kunz, C.; Meyer, C.; Collado, M. C.; Geiger, L.; García-Mantrana, I.; Bertua-Ríos, B.; Martínez-Costa, C.; Borsch, C.; Rudloff, S., Influence of Gestational Age, Secretor, and Lewis Blood Group Status on the Oligosaccharide Content of Human Milk. *J Pediatr Gastroenterol Nutr* **2017,** *64* (5), 789-798.

24. Xu, G.; Davis, J. C.; Goonatilleke, E.; Smilowitz, J. T.; German, J. B.; Lebrilla, C. B., Absolute Quantitation of Human Milk Oligosaccharides Reveals Phenotypic Variations during Lactation. *J Nutr* **2017,** *147* (1), 117-124.

25. Smilowitz, J. T.; Lebrilla, C. B.; Mills, D. A.; German, J. B.; Freeman, S. L., Breast milk oligosaccharides: structure-function relationships in the neonate. *Annu Rev Nutr* **2014,** *34*, 143-69.

26. Bode, L., Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* **2012,** *22* (9), 1147-62.

27. Kunz, C.; Rudloff, S.; Schad, W.; Braun, D., Lactose-derived oligosaccharides in the milk of elephants: comparison with human milk. *Br J Nutr* **1999,** *82* (5), 391-9.

28. Newburg, D. S.; Shen, Z.; Warren, C. D., Quantitative analysis of human milk oligosaccharides by capillary electrophoresis. *Adv Exp Med Biol* **2000,** *478*, 381-2.

29. Gopal, P. K.; Gill, H. S., Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br J Nutr* **2000,** *84 Suppl 1*, S69-74.

30. Bode, L.; Kuhn, L.; Kim, H. Y.; Hsiao, L.; Nissan, C.; Sinkala, M.; Kankasa, C.; Mwiya, M.; Thea, D. M.; Aldrovandi, G. M., Human milk oligosaccharide concentration and risk of postnatal transmission of HIV through breastfeeding. *Am J Clin Nutr* **2012,** *96* (4), 831-9.

31. Lonnerdal, B., Human milk proteins: key components for the biological activity of human milk. *Adv Exp Med Biol* **2004,** *554*, 11-25.

32. Allen, L. H., B vitamins in breast milk: relative importance of maternal status and intake, and effects on infant status and function. *Adv Nutr* **2012,** *3* (3), 362-9.

33. Ruiz, L.; Espinosa-Martos, I.; Garcia-Carral, C.; Manzano, S.; McGuire, M. K.; Meehan, C. L.; McGuire, M. A.; Williams, J. E.; Foster, J.; Sellen, D. W.; Kamau-Mbuthia, E. W.; Kamundia, E. W.; Mbugua, S.; Moore, S. E.; Kvist, L. J.; Otoo, G. E.; Lackey, K. A.; Flores, K.; Pareja, R. G.; Bode, L.; Rodriguez, J. M., What's Normal? Immune Profiling of Human Milk from Healthy Women Living in Different Geographical and Socioeconomic Settings. *Front Immunol* **2017,** *8*, 696.

34. Hirai, C.; Ichiba, H.; Saito, M.; Shintaku, H.; Yamano, T.; Kusuda, S., Trophic effect of multiple growth factors in amniotic fluid or human milk on cultured human fetal small intestinal cells. *J Pediatr Gastroenterol Nutr* **2002,** *34* (5), 524-8.

35. Wagner, C. L.; Taylor, S. N.; Johnson, D., Host factors in amniotic fluid and breast milk that contribute to gut maturation. *Clin Rev Allergy Immunol* **2008,** *34* (2), 191-204.

36. Chang, C. J.; Chao, J. C., Effect of human milk and epidermal growth factor on growth of human intestinal Caco-2 cells. *J Pediatr Gastroenterol Nutr* **2002,** *34* (4), 394-401.

37. Rodrigues, D. M.; Li, A. Y.; Nair, D. G.; Blennerhassett, M. G., Glial cell line-derived neurotrophic factor is a key neurotrophin in the postnatal enteric nervous system. *Neurogastroenterol Motil* **2011,** *23* (2), e44-56.

38. Boesmans, W.; Gomes, P.; Janssens, J.; Tack, J.; Vanden Berghe, P., Brain-derived neurotrophic factor amplifies neurotransmitter responses and promotes synaptic communication in the enteric nervous system. *Gut* **2008,** *57* (3), 314-22.

39. Li, R.; Xia, W.; Zhang, Z.; Wu, K., S100B protein, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor in human milk. *Plos One* **2011,** *6* (6), e21663.

40. Fichter, M.; Klotz, M.; Hirschberg, D. L.; Waldura, B.; Schofer, O.; Ehnert, S.; Schwarz, L. K.; Ginneken, C. V.; Schafer, K. H., Breast milk contains relevant neurotrophic factors and cytokines for enteric nervous system development. *Mol Nutr Food Res* **2011,** *55* (10), 1592-6.

41. Loui, A.; Eilers, E.; Strauss, E.; Pohl-Schickinger, A.; Obladen, M.; Koehne, P., Vascular Endothelial Growth Factor (VEGF) and soluble VEGF receptor 1 (sFlt-1) levels in early and mature human milk from mothers of preterm versus term infants. *J Hum Lact* **2012,** *28* (4), 522-8.

42. Kett, J. C., Anemia in infancy. *Pediatr Rev* **2012,** *33* (4), 186-7.

43. Soubasi, V.; Kremenopoulos, G.; Diamanti, E.; Tsantali, C.; Sarafidis, K.; Tsakiris, D., Follow-up of very low birth weight infants after erythropoietin treatment to prevent anemia of prematurity. *J Pediatr* **1995,** *127* (2), 291-7.

44. Shiou, S. R.; Yu, Y.; Chen, S.; Ciancio, M. J.; Petrof, E. O.; Sun, J.; Claud, E. C., Erythropoietin protects intestinal epithelial barrier function and lowers the incidence of experimental neonatal necrotizing enterocolitis. *J Biol Chem* **2011,** *286* (14), 12123-32.

45. Claud, E. C.; Savidge, T.; Walker, W. A., Modulation of human intestinal epithelial cell IL-8 secretion by human milk factors. *Pediatr Res* **2003,** *53* (3), 419-25.

46. Savino, F.; Liguori, S. A., Update on breast milk hormones: leptin, ghrelin and adiponectin. *Clin Nutr* **2008,** *27* (1), 42-7.

47. Savino, F.; Sorrenti, M.; Benetti, S.; Lupica, M. M.; Liguori, S. A.; Oggero, R., Resistin and leptin in breast milk and infants in early life. *Early Hum Dev* **2012,** *88* (10), 779-82.

48. Dundar, N. O.; Dundar, B.; Cesur, G.; Yilmaz, N.; Sutcu, R.; Ozguner, F., Ghrelin and adiponectin levels in colostrum, cord blood and maternal serum. *Pediatr Int* **2010,** *52* (4), 622-5.

49. Cesur, G.; Ozguner, F.; Yilmaz, N.; Dundar, B., The relationship between ghrelin and adiponectin levels in breast milk and infant serum and growth of infants during early postnatal life. *J Physiol Sci* **2012,** *62* (3), 185-90.

50. Werner, S.; Widström, A. M.; Wahlberg, V.; Eneroth, P.; Winberg, J., Immunoreactive calcitonin in maternal milk and serum in relation to prolactin and neurotensin. *Early Hum Dev* **1982,** *6* (1), 77-82.

51. Cacho, N. T.; Lawrence, R. M., Innate Immunity and Breast Milk. *Front Immunol* **2017,** *8*, 584.

52. Ichikawa, M.; Sugita, M.; Takahashi, M.; Satomi, M.; Takeshita, T.; Araki, T.; Takahashi, H., Breast milk macrophages spontaneously produce granulocyte-macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. *Immunology* **2003,** *108* (2), 189-95.

53. Hassiotou, F.; Beltran, A.; Chetwynd, E.; Stuebe, A. M.; Twigger, A. J.; Metzger, P.; Trengove, N.; Lai, C. T.; Filgueira, L.; Blancafort, P.; Hartmann, P. E., Breastmilk is a novel source of stem cells with multilineage differentiation potential. *Stem Cells* **2012,** *30* (10), 2164-74.

54. Hassiotou, F.; Hepworth, A. R.; Metzger, P.; Tat Lai, C.; Trengove, N.; Hartmann, P. E.; Filgueira, L., Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. *Clin Transl Immunology* **2013,** *2* (4), e3.

55. Birch, H.; Giles, C., Immune response component images. In *Immune System*, Stubberfield, L.; Giles, C.; Freeman, T.; Reddick, R., Eds. Big Picture: 2015; Vol. Issue 21, January 2015.

56. Garofalo, R., Cytokines in human milk. *J Pediatr* **2010,** *156* (2 Suppl), S36-40.

57. Kalliomaki, M.; Ouwehand, A.; Arvilommi, H.; Kero, P.; Isolauri, E., Transforming growth factor-beta in breast milk: a potential regulator of atopic disease at an early age. *J Allergy Clin Immunol* **1999,** *104* (6), 1251-7.

58. Saito, S.; Yoshida, M.; Ichijo, M.; Ishizaka, S.; Tsujii, T., Transforming growth factor-beta (TGF- β) in human milk. *Clin Exp Immunol* **1993,** *94* (1), 220-4.

59. Verhasselt, V., Neonatal tolerance under breastfeeding influence: the presence of allergen and transforming growth factor-beta in breast milk protects the progeny from allergic asthma. *J Pediatr* **2010,** *156* (2 Suppl), S16-20.

60. Faria, A. M.; Weiner, H. L., Oral tolerance and TGF-beta-producing cells. *Inflamm Allergy Drug Targets* **2006,** *5* (3), 179-90.

61. Ando, T.; Hatsushika, K.; Wako, M.; Ohba, T.; Koyama, K.; Ohnuma, Y.; Katoh, R.; Ogawa, H.; Okumura, K.; Luo, J.; Wyss-Coray, T.; Nakao, A., Orally administered TGF-beta is biologically active in the intestinal mucosa and enhances oral tolerance. *J Allergy Clin Immunol* **2007,** *120* (4), 916-23.

62. Gersting, J. A.; Christensen, R. D.; Calhoun, D. A., Effects of enterally administering granulocyte colony-stimulating factor to suckling mice. *Pediatr Res* **2004,** *55* (5), 802-6.

63. Aspinall, R.; Prentice, A. M.; Ngom, P. T., Interleukin 7 from maternal milk crosses the intestinal barrier and modulates T-cell development in offspring. *Plos One* **2011,** *6* (6), e20812.

64. Ebert, E. C., Human intestinal intraepithelial lymphocytes have potent chemotactic activity. *Gastroenterology* **1995,** *109* (4), 1154-9.

65. Omari, K. M.; Lutz, S. E.; Santambrogio, L.; Lira, S. A.; Raine, C. S., Neuroprotection and remyelination after autoimmune demyelination in mice that inducibly overexpress CXCL1. *Am J Pathol* **2009,** *174* (1), 164-76.

66. Vries, M. H.; Wagenaar, A.; Verbruggen, S. E.; Molin, D. G.; Dijkgraaf, I.; Hackeng, T. H.; Post, M. J., CXCL1 promotes arteriogenesis through enhanced monocyte recruitment into the peri-collateral space. *Angiogenesis* **2015,** *18* (2), 163-71.

67. Cocchi, F.; DeVico, A. L.; Garzino-Demo, A.; Arya, S. K.; Gallo, R. C.; Lusso, P., Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **1995,** *270* (5243), 1811-5.

68. Jarvinen, K. M.; Laine, S. T.; Jarvenpaa, A. L.; Suomalainen, H. K., Does low IgA in human milk predispose the infant to development of cow's milk allergy? *Pediatr Res* **2000,** *48* (4), 457-62.

69. Savilahti, E.; Siltanen, M.; Kajosaari, M.; Vaarala, O.; Saarinen, K. M., IgA antibodies, TGF-beta1 and -beta2, and soluble CD14 in the colostrum and development of atopy by age 4. *Pediatr Res* **2005,** *58* (6), 1300-5.

70. Mantis, N. J.; Rol, N.; Corthesy, B., Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol* **2011,** *4* (6), 603-11.

71. Goldman, A. S., The immune system of human milk: antimicrobial, antiinflammatory and immunomodulating properties. *Pediatr Infect Dis J* **1993,** *12* (8), 664-71.

72. Lawrence, R. M.; Pane, C. A., Human breast milk: current concepts of immunology and infectious diseases. *Curr Probl Pediatr Adolesc Health Care* **2007,** *37* (1), 7-36.

73. Le Doare, K.; Kampmann, B., Breast milk and Group B *streptococcal* infection: vector of transmission or vehicle for protection? *Vaccine* **2014,** *32* (26), 3128-32.

74. Heiman, H. S.; Weisman, L. E., Transplacental or enteral transfer of maternal immunization-induced antibody protects suckling rats from type III group B *streptococcal* infection. *Pediatr Res* **1989,** *26* (6), 629-32.

75. Weisman, L. E.; Dobson, F. M., The potential impact of group B *streptococcal* antibodies in breast milk. *Adv Exp Med Biol* **1991,** *310*, 345-51.

76. Lagergard, T.; Thiringer, K.; Wassen, L.; Schneerson, R.; Trollfors, B., Isotype composition of antibodies to *streptococcus* group B type III polysaccharide and to tetanus toxoid in maternal, cord blood sera and in breast milk. *Eur J Pediatr* **1992,** *151* (2), 98-102.

77. Gray, B. M.; Egan, M. L.; Pritchard, D. G., Specificity of monoclonal antibodies against group B *streptococcus* type II and inhibition of their binding by human secretions. *Pediatr Res* **1988,** *24* (1), 68-72.

78. Beljaars, L.; van der Strate, B. W.; Bakker, H. I.; Reker-Smit, C.; van Loenen-Weemaes, A. M.; Wiegmans, F. C.; Harmsen, M. C.; Molema, G.; Meijer, D. K., Inhibition of cytomegalovirus infection by lactoferrin in vitro and in vivo. *Antiviral Res* **2004,** *63* (3), 197-208.

79. Kuipers, M. E.; de Vries, H. G.; Eikelboom, M. C.; Meijer, D. K.; Swart, P. J., Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical Candida isolates. *Antimicrob Agents Chemother* **1999,** *43* (11), 2635-41.

80. Leitch, E. C.; Willcox, M. D., Lactoferrin increases the susceptibility of S. epidermidis biofilms to lysozyme and vancomycin. *Curr Eye Res* **1999,** *19* (1), 12-9.

81. Legrand, D., Overview of Lactoferrin as a Natural Immune Modulator. *J Pediatr* **2016,** *173 Suppl*, S10-5.

82. Newburg, D. S.; Peterson, J. A.; Ruiz-Palacios, G. M.; Matson, D. O.; Morrow, A. L.; Shults, J.; Guerrero, M. d. L.; Chaturvedi, P.; Newburg, S. O.; Scallan, C. D.; Taylor, M.

R.; Ceriani, R. L.; Pickering, L. K., Role of human-milk lactadherin in protectoin against symptomatic rotavirus infection. *The Lancet* **1998,** *351* (9110), 1160-1164.

83. Hanayama, R.; Tanaka, M.; Miwa, K.; Shinohara, A.; Iwamatsu, A.; Nagata, S., Identification of a factor that links apoptotic cells to phagocytes. *Nature* **2002,** *417* (6885), 182-7.

84. Shi, J.; Heegaard, C. W.; Rasmussen, J. T.; Gilbert, G. E., Lactadherin binds selectively to membranes containing phosphatidyl-L-serine and increased curvature. *Biochim Biophys Acta* **2004,** *1667* (1), 82-90.

85. Liu, B.; Newburg, D. S., Human milk glycoproteins protect infants against human pathogens. *Breastfeed Med* **2013,** *8* (4), 354-62.

86. McGuire, M. K.; McGuire, M. A., Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. *Curr Opin Biotechnol* **2017,** *44*, 63-68.

87. Hunt, K. M.; Foster, J. A.; Forney, L. J.; Schutte, U. M.; Beck, D. L.; Abdo, Z.; Fox, L. K.; Williams, J. E.; McGuire, M. K.; McGuire, M. A., Characterization of the diversity and temporal stability of bacterial communities in human milk. *Plos One* **2011,** *6* (6), e21313.

88. Cabrera-Rubio, R.; Collado, M. C.; Laitinen, K.; Salminen, S.; Isolauri, E.; Mira, A., The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr* **2012,** *96* (3), 544-51.

89. Urbaniak, C.; Angelini, M.; Gloor, G. B.; Reid, G., Human milk microbiota profiles in relation to birthing method, gestation and infant gender. *Microbiome* **2016,** *4*, 1.

90. McCracken, V. J.; Lorenz, R. G., The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cell Microbiol* **2001,** *3* (1), 1-11.

91. Cacho, N.; Neu, J., Manipulation of the intestinal microbiome in newborn infants. *Adv Nutr* **2014,** *5* (1), 114-8.

92. Backhed, F.; Roswall, J.; Peng, Y.; Feng, Q.; Jia, H.; Kovatcheva-Datchary, P.; Li, Y.; Xia, Y.; Xie, H.; Zhong, H.; Khan, M. T.; Zhang, J.; Li, J.; Xiao, L.; Al-Aama, J.; Zhang, D.; Lee, Y. S.; Kotowska, D.; Colding, C.; Tremaroli, V.; Yin, Y.; Bergman, S.; Xu, X.; Madsen, L.; Kristiansen, K.; Dahlgren, J.; Wang, J., Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* **2015,** *17* (6), 852.

93. Fernandez, L.; Langa, S.; Martin, V.; Maldonado, A.; Jimenez, E.; Martin, R.; Rodriguez, J. M., The human milk microbiota: origin and potential roles in health and disease. *Pharmacol Res* **2013,** *69* (1), 1-10.

94. Walker, W. A.; Iyengar, R. S., Breast milk, microbiota, and intestinal immune homeostasis. *Pediatr Res* **2015,** *77* (1-2), 220-8.

95. Jones, C. A., Maternal transmission of infectious pathogens in breast milk. *J Paediatr Child Health* **2001,** *37* (6), 576-82.

96. Schwartz, S.; Friedberg, I.; Ivanov, I. V.; Davidson, L. A.; Goldsby, J. S.; Dahl, D. B.; Herman, D.; Wang, M.; Donovan, S. M.; Chapkin, R. S., A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in immune response. *Genome Biol* **2012,** *13* (4), r32.

97. Gomez-Gallego, C.; Garcia-Mantrana, I.; Salminen, S.; Collado, M. C., The human milk microbiome and factors influencing its composition and activity. *Semin Fetal Neonatal Med* **2016,** *21* (6), 400-405.

98. Fitzstevens, J. L.; Smith, K. C.; Hagadorn, J. I.; Caimano, M. J.; Matson, A. P.; Brownell, E. A., Systematic Review of the Human Milk Microbiota. *Nutr Clin Pract* **2017,** *32* (3), 354-364.

99. Newburg, D. S., Glycobiology of human milk. *Biochemistry (Mosc)* **2013,** *78* (7), 771-85.

100. Engfer, M. B.; Stahl, B.; Finke, B.; Sawatzki, G.; Daniel, H., Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* **2000,** *71* (6), 1589-96.

101. Gnoth, M. J.; Kunz, C.; Kinne-Saffran, E.; Rudloff, S., Human milk oligosaccharides are minimally digested in vitro. *J Nutr* **2000,** *130* (12), 3014-20.

102. Bode, L., Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology* **2012,** *22* (9), 1147-1162.

103. Hinde, K.; Lewis, Z. T., Microbiota. Mother's littlest helpers. *Science* **2015,** *348* (6242), 1427-8.

104. Mueller, N. T.; Bakacs, E.; Combellick, J.; Grigoryan, Z.; Dominguez-Bello, M. G., The infant microbiome development: mom matters. *Trends Mol Med* **2015,** *21* (2), 109-17.

105. Asakuma, S.; Hatakeyama, E.; Urashima, T.; Yoshida, E.; Katayama, T.; Yamamoto, K.; Kumagai, H.; Ashida, H.; Hirose, J.; Kitaoka, M., Physiology of consumption of human milk oligosaccharides by infant gut-associated *bifidobacteria*. *J Biol Chem* **2011,** *286* (40), 34583-92.

106. Bode, L., The functional biology of human milk oligosaccharides. *Early Hum Dev* **2015,** *91* (11), 619-622.

107. Morrow, A. L.; Ruiz-Palacios, G. M.; Altaye, M.; Jiang, X.; Guerrero, M. L.; Meinzen-Derr, J. K.; Farkas, T.; Chaturvedi, P.; Pickering, L. K.; Newburg, D. S., Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J Pediatr* **2004,** *145* (3), 297-303.

108. Morrow, A. L.; Ruiz-Palacios, G. M.; Jiang, X.; Newburg, D. S., Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *J Nutr* **2005,** *135* (5), 1304-7.

109. Stepans, M. B.; Wilhelm, S. L.; Hertzog, M.; Rodehorst, T. K.; Blaney, S.; Clemens, B.; Polak, J. J.; Newburg, D. S., Early consumption of human milk oligosaccharides is inversely related to subsequent risk of respiratory and enteric disease in infants. *Breastfeed Med* **2006,** *1* (4), 207-15.

110. Etzold, S.; Bode, L., Glycan-dependent viral infection in infants and the role of human milk oligosaccharides. *Curr Opin Virol* **2014,** *7*, 101-7.

111. Wang, B., Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Adv Nutr* **2012,** *3* (3), 465S-72S.

112. Wang, B., Sialic acid is an essential nutrient for brain development and cognition. *Annu Rev Nutr* **2009,** *29*, 177-222.

113. He, Y.; Liu, S.; Kling, D. E.; Leone, S.; Lawlor, N. T.; Huang, Y.; Feinberg, S. B.; Hill, D. R.; Newburg, D. S., The human milk oligosaccharide 2'-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. *Gut* **2016,** *65* (1), 33-46.

114. Jantscher-Krenn, E.; Zherebtsov, M.; Nissan, C.; Goth, K.; Guner, Y. S.; Naidu, N.; Choudhury, B.; Grishin, A. V.; Ford, H. R.; Bode, L., The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats. *Gut* **2012,** *61* (10), 1417-25.

115. Chen, G. Y.; Brown, N. K.; Wu, W.; Khedri, Z.; Yu, H.; Chen, X.; van de Vlekkert, D.; D'Azzo, A.; Zheng, P.; Liu, Y., Broad and direct interaction between TLR and Siglec families of pattern recognition receptors and its regulation by Neu1. *eLIFE* **2014,** *3*, e04066.

116. Niño, D. F.; Sodhi, C. P.; Hackam, D. J., Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. *Nat Rev Gastroenterol Hepatol* **2016,** *13* (10), 590-600.

117. Kunz, C., Historical aspects of human milk oligosaccharides. *Adv Nutr* **2012,** *3* (3), 430S-9S.

118. Polonowski, M.; Lespagnol, A., Sur la nature glucidique de la substance lévogyre du lait de femme. *Bull Soc Biol* **1929,** *101*, 61-63.

119. Polonowski, M.; Lespagnol, A., Sur deux nouveaux sucres du lait de femme, le gynolactose et l'allolactose. *C R Acad Sci* **1931,** *192*, 1319.

120. Polonowski, M.; Montreuil, J., Etude chromatographique des polyosides du lait de Femme. *C R Acad Sci Paris* **1954,** *238*, 2263-2264.

121. Kuhn, R.; Baer, H. H.; Bister, W.; Brossmer, R.; Gauhe, A.; Haas, H. J.; Haber, F.; Krüger, G.; Tiedemann, H.; Weiser, D., Aminozucker. *Angew Chem Int Ed* **1957,** *69* (1-2), 23-33.

122. Kuhn, R., [Oligosaccharides of milk]. *Bull Soc Chim Biol (Paris)* **1958,** *40* (2-3), 297-314.

123. Kuhn, R.; Baer, H. H., Die Konstitution der Lacto-N-tetraose. *Chem Ber* **1956,** *89* (2), 504-511.

124. Montreuil, J., [The glucides of milk]. *Bull Soc Chim Biol (Paris)* **1960,** *42*, 1399-427.

125. Grimmonprez, L.; Montreuil, J., [The glycan fraction of the total glycosphingolipids of the human milk fat globule membrane]. *Biochimie* **1977,** *59* (11-12), 899-907.

126. Kuhn, R.; Baer, H. H.; Gauhe, A., Kristallisation und Konstitutionsermittlung der Lacto-N-fucopentaose I. *Chem Ber* **1956,** *89* (11), 2514-2523.

127. Kuhn, R.; Baer, H. H.; Gauhe, A., Die Konstitution der Lacto-N-fucopentaose II. Ein Beitrag zur Spezifität der Blutgruppensubstanz Lea. *Chem Ber* **1958,** *91* (2), 364-374.

128. Kuhn, R.; Gauhe, A., Über ein kristallisiertes, Lea-aktives Hexasaccharid aus Frauenmilch. *Chem Ber* **1960,** *93* (3), 647-651.

129. Kuhn, R.; Gauhe, A., Über die Lacto-Difuco-Tetraose Der Frauenmilch. Ein Beitrag zur Strukturspezifität der Blutgruppensubstanz Le. *Justus Liebigs Ann Chem* **1958,** *611* (1), 249-253.

130. Kuhn, R.; Gauhe, A., Über drei saure pentasaccharide aus Frauenmilch. *Chem Ber* **1962,** *95* (2), 513-517.

131. Kobata, A., Structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B Phys Biol Sci* **2010,** *86* (7), 731-47.

132. Grollman, E. F.; Ginsburg, V., Correlation between Secretor Status and Occurrence of 2'-Fucosyllactose in Human Milk. *Biochem Bioph Res Co* **1967,** *28* (1), 50-&.

133. Grollman, E. F.; Kobata, A.; Ginsburg, V., Enzymatic basis of blood types in man. *Ann N Y Acad Sci* **1970,** *169* (1), 153-60.

134. Grollman, E. F.; Kobata, A.; Ginsburg, V., An enzymatic basis for Lewis blood types in man. *J Clin Invest* **1969,** *48* (8), 1489-94.

135. Kobata, A.; Ginsburg, V.; Tsuda, M., Oligosaccharides of human milk. I. Isolation and characterization. *Arch Biochem Biophys* **1969,** *130* (1), 509-13.

136. Kobata, A.; Ginsburg, V., Oligosaccharides of human milk. II. Isolation and characterization of a new pentasaccharide, lacto-N-fucopentaose 3. *J Biol Chem* **1969,** *244* (20), 5496-502.

137. Kobata, A.; Ginsburg, V., Oligosaccharides of human milk. 3. Isolation and characterization of a new hexasaccharide, lacto-N-hexaose. *J Biol Chem* **1972,** *247* (5), 1525-9.

138. Kobata, A.; Ginsburg, V., Oligosaccharides of human milk. IV. Isolation and characterization of a new hexasaccharide, lacto-N-neohexaose. *Arch Biochem Biophys* **1972,** *150* (1), 273-81.

139. Egge, H.; Dell, A.; Von Nicolai, H., Fucose containing oligosaccharides from human milk. I. Separation and identification of new constituents. *Arch Biochem Biophys* **1983,** *224* (1), 235-53.

140. Newburg, D. S.; Neubauer, S. H., CHAPTER 4 - Carbohydrates in Milks: Analysis, Quantities, and Significance A2 - Jensen, Robert G. In *Handbook of Milk Composition*, Academic Press: San Diego, 1995; pp 273-349.

141. Wu, S.; Tao, N.; German, J. B.; Grimm, R.; Lebrilla, C. B., Development of an annotated library of neutral human milk oligosaccharides. *J Proteome Res* **2010,** *9* (8), 4138-51.

142. Wu, S.; Grimm, R.; German, J. B.; Lebrilla, C. B., Annotation and structural analysis of sialylated human milk oligosaccharides. *J Proteome Res* **2011,** *10* (2), 856-68.

143. Urashima, T.; Kitaoka, M.; Asakuma, S.; Messer, M., Milk Oligosaccharides. In *Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts and Minor Constituents*, McSweeney, P.; Fox, P. F., Eds. Springer New York: New York, NY, 2009; pp 295-349.

144. Chen, X., Human Milk Oligosaccharides (HMOS): Structure, Function, and Enzyme-Catalyzed Synthesis. *Adv Carbohydr Chem Biochem* **2015,** *72*, 113-90.

145. Newburg, D. S., Oligosaccharides in human milk and bacterial colonization. *J Pediatr Gastroenterol Nutr* **2000,** *30 Suppl 2*, S8-17.

146. Stahl, B.; Thurl, S.; Zeng, J.; Karas, M.; Hillenkamp, F.; Steup, M.; Sawatzki, G., Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* **1994,** *223* (2), 218-26.

147. Chaturvedi, P.; Warren, C. D.; Altaye, M.; Morrow, A. L.; Ruiz-Palacios, G.; Pickering, L. K.; Newburg, D. S., Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* **2001,** *11* (5), 365-72.

148. Davidson, B.; Meinzen-Derr, J. K.; Wagner, C. L.; Newburg, D. S.; Morrow, A. L., Fucosylated oligosaccharides in human milk in relation to gestational age and stage of lactation. *Adv Exp Med Biol* **2004,** *554*, 427-30.

149. Bao, Y.; Zhu, L.; Newburg, D. S., Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. *Anal Biochem* **2007,** *370* (2), 206-14.

150. Gabrielli, O.; Zampini, L.; Galeazzi, T.; Padella, L.; Santoro, L.; Peila, C.; Giuliani, F.; Bertino, E.; Fabris, C.; Coppa, G. V., Preterm milk oligosaccharides during the first month of lactation. *Pediatrics* **2011,** *128* (6), e1520-31.

151. Ninonuevo, M. R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R. E.; Clowers, B. H.; German, J. B.; Freeman, S. L.; Killeen, K.; Grimm, R.; Lebrilla, C. B., A strategy for annotating the human milk glycome. *J Agric Food Chem* **2006,** *54* (20), 7471-80.

152. Tao, N.; DePeters, E. J.; Freeman, S.; German, J. B.; Grimm, R.; Lebrilla, C. B., Bovine milk glycome. *J Dairy Sci* **2008,** *91* (10), 3768-78.

153. Tao, N.; DePeters, E. J.; German, J. B.; Grimm, R.; Lebrilla, C. B., Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *J Dairy Sci* **2009,** *92* (7), 2991-3001.

154. Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S., Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr* **2000,** *20*, 699-722.

155. Kudo, T.; Iwasaki, H.; Nishihara, S.; Shinya, N.; Ando, T.; Narimatsu, I.; Narimatsu, H., Molecular genetic analysis of the human Lewis histo-blood group system. II. Secretor gene inactivation by a novel single missense mutation A385T in Japanese nonsecretor individuals. *J Biol Chem* **1996,** *271* (16), 9830-7.

156. Oriol, R.; Le Pendu, J.; Mollicone, R., Genetics of ABO, H, Lewis, X and related antigens. *Vox Sang* **1986,** *51* (3), 161-71.

157. Bode, L., Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev* **2009,** *67 Suppl 2*, S183-91.

158. Ramakrishnan, B.; Boeggeman, E.; Qasba, P. K., β-1,4-Galactosyltransferase and lactose synthase: molecular mechanical devices. *Biochem Biophys Res Commun* **2002,** *291* (5), 1113-8.

159. Brew, K.; Hill, R. L., Lactose biosynthesis. *Rev Physiol Biochem Pharmacol* **1975,** *72*, 105-58.

160. Kobata, A., Possible application of milk oligosaccharides for drug development. *Chang Gung Med J* **2003,** *26* (9), 621-36.

161. Appert, H. E.; Rutherford, T. J.; Tarr, G. E.; Thomford, N. R.; McCorquodale, D. J., Isolation of galactosyltransferase from human milk and the determination of its N-terminal amino acid sequence. *Biochem Biophys Res Commun* **1986,** *138* (1), 224-9.

162. Endo, T.; Amano, J.; Berger, E. G.; Kobata, A., Structure identification of the complex-type, asparagine-linked sugar chains of β-D-galactosyl-transferase purified from human milk. *Carbohydr Res* **1986,** *150*, 241-63.

163. Prieels, J. P.; Monnom, D.; Dolmans, M.; Beyer, T. A.; Hill, R. L., Co-purification of the Lewis blood group N-acetylglucosaminide alpha 1 goes to 4 fucosyltransferase and an N-acetylglucosaminide alpha 1 goes to 3 fucosyltransferase from human milk. *J Biol Chem* **1981,** *256* (20), 10456-63.

164. Eppenberger-Castori, S.; Lotscher, H.; Finne, J., Purification of the N-acetylglucosaminide α(1-3/4)fucosyltransferase of human milk. *Glycoconj J* **1989,** *6* (1), 101-14.

165. Johnson, P. H.; Donald, A. S.; Feeney, J.; Watkins, W. M., Reassessment of the acceptor specificity and general properties of the Lewis blood-group gene associated α-3/4-fucosyltransferase purified from human milk. *Glycoconj J* **1992,** *9* (5), 251-64.

166. Johnson, P. H.; Watkins, W. M., Purification of the Lewis blood-group gene associated alpha-3/4-fucosyltransferase from human milk: an enzyme transferring fucose primarily to type 1 and lactose-based oligosaccharide chains. *Glycoconj J* **1992,** *9* (5), 241-9.

167. Grabarics, M.; Csernak, O.; Balogh, R.; Beni, S., Analytical characterization of human milk oligosaccharides - potential applications in pharmaceutical analysis. *J Pharm Biomed Anal* **2017,** *146*, 168-178.

168. Chaturvedi, P.; Warren, C. D.; Ruiz-Palacios, G. M.; Pickering, L. K.; Newburg, D. S., Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. *Anal Biochem* **1997,** *251* (1), 89-97.

169. Sumiyoshi, W.; Urashima, T.; Nakamura, T.; Arai, I.; Saito, T.; Tsumura, N.; Wang, B.; Brand-Miller, J.; Watanabe, Y.; Kimura, K., Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. *Br J Nutr* **2003,** *89* (1), 61-69.

170. Leo, F.; Asakuma, S.; Nakamura, T.; Fukuda, K.; Senda, A.; Urashima, T., Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high-performance liquid chromatography. *J Chromatogr A* **2009,** *1216* (9), 1520-3.

171. Kunz, C.; Rudloff, S.; Hintelmann, A.; Pohlentz, G.; Egge, H., High-pH anion-exchange chromatography with pulsed amperometric detection and molar response factors of human milk oligosaccharides. *J Chromatogr B Biomed Appl* **1996,** *685* (2), 211-21.

172. Thurl, S.; Muller-Werner, B.; Sawatzki, G., Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. *Anal Biochem* **1996,** *235* (2), 202-6.

173. West, C.; Elfakir, C.; Lafosse, M., Porous graphitic carbon: a versatile stationary phase for liquid chromatography. *J Chromatogr A* **2010,** *1217* (19), 3201-16.

174. Balogh, R.; Jankovics, P.; Beni, S., Qualitative and quantitative analysis of N-acetyllactosamine and lacto-N-biose, the two major building blocks of human milk oligosaccharides in human milk samples by high-performance liquid chromatography-tandem mass spectrometry using a porous graphitic carbon column. *J Chromatogr A* **2015,** *1422*, 140-146.

175. Bao, Y.; Chen, C.; Newburg, D. S., Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. *Anal Biochem* **2013,** *433* (1), 28-35.

176. Charlwood, J.; Tolson, D.; Dwek, M.; Camilleri, P., A detailed analysis of neutral and acidic carbohydrates in human milk. *Anal Biochem* **1999,** *273* (2), 261-77.

177. Fong, B.; Ma, K.; McJarrow, P., Quantification of bovine milk oligosaccharides using liquid chromatography-selected reaction monitoring-mass spectrometry. *J Agric Food Chem* **2011,** *59* (18), 9788-95.

178. Marino, K.; Lane, J. A.; Abrahams, J. L.; Struwe, W. B.; Harvey, D. J.; Marotta, M.; Hickey, R. M.; Rudd, P. M., Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography. *Glycobiology* **2011,** *21* (10), 1317-30.

179. Galeotti, F.; Coppa, G. V.; Zampini, L.; Maccari, F.; Galeazzi, T.; Padella, L.; Santoro, L.; Gabrielli, O.; Volpi, N., On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone. *Anal Biochem* **2012,** *430* (1), 97-104.

180. Balogh, R.; Szarka, S.; Beni, S., Determination and quantification of 2'-O-fucosyllactose and 3-O-fucosyllactose in human milk by GC-MS as O-trimethylsilyl-oxime derivatives. *J Pharm Biomed Anal* **2015,** *115*, 450-6.

181. Monti, L.; Cattaneo, T. M.; Orlandi, M.; Curadi, M. C., Capillary electrophoresis of sialylated oligosaccharides in milk from different species. *J Chromatogr A* **2015,** *1409*, 288-91.

182. Bao, Y.; Newburg, D. S., Capillary electrophoresis of acidic oligosaccharides from human milk. *Electrophoresis* **2008,** *29* (12), 2508-15.

183. Song, J. F.; Weng, M. Q.; Wu, S. M.; Xia, Q. C., Analysis of neutral saccharides in human milk derivatized with 2-aminoacridone by capillary electrophoresis with laser-induced fluorescence detection. *Anal Biochem* **2002,** *304* (1), 126-9.

184. Albrecht, S.; Schols, H. A.; van den Heuvel, E. G.; Voragen, A. G.; Gruppen, H., CE-LIF-MS n profiling of oligosaccharides in human milk and feces of breast-fed babies. *Electrophoresis* **2010,** *31* (7), 1264-73.

185. Stahl, B.; Thurl, S.; Henker, J.; Siegel, M.; Finke, B.; Sawatzki, G., Detection of four human milk groups with respect to Lewis-blood-group-dependent oligosaccharides by serologic and chromatographic analysis. *Adv Exp Med Biol* **2001,** *501*, 299-306.

186. Blank, D.; Gebhardt, S.; Maass, K.; Lochnit, G.; Dotz, V.; Blank, J.; Geyer, R.; Kunz, C., High-throughput mass finger printing and Lewis blood group assignment of human milk oligosaccharides. *Anal Bioanal Chem* **2011,** *401* (8), 2495-510.

187. Totten, S. M.; Zivkovic, A. M.; Wu, S.; Ngyuen, U.; Freeman, S. L.; Ruhaak, L. R.; Darboe, M. K.; German, J. B.; Prentice, A. M.; Lebrilla, C. B., Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *J Proteome Res* **2012,** *11* (12), 6124-33.

188. van Leeuwen, S. S.; Schoemaker, R. J.; Gerwig, G. J.; van Leusen-van Kan, E. J.; Dijkhuizen, L.; Kamerling, J. P., Rapid milk group classification by 1H NMR analysis of Le and H epitopes in human milk oligosaccharide donor samples. *Glycobiology* **2014,** *24* (8), 728-39.

189. Andreas, N. J.; Al-Khalidi, A.; Jaiteh, M.; Clarke, E.; Hyde, M. J.; Modi, N.; Holmes, E.; Kampmann, B.; Mehring Le Doare, K., Role of human milk oligosaccharides in Group B *Streptococcus* colonisation. *Clin Transl Immunology* **2016,** *5* (8), e99.

Appendix A1:

Spectra Relevant to Chapter II

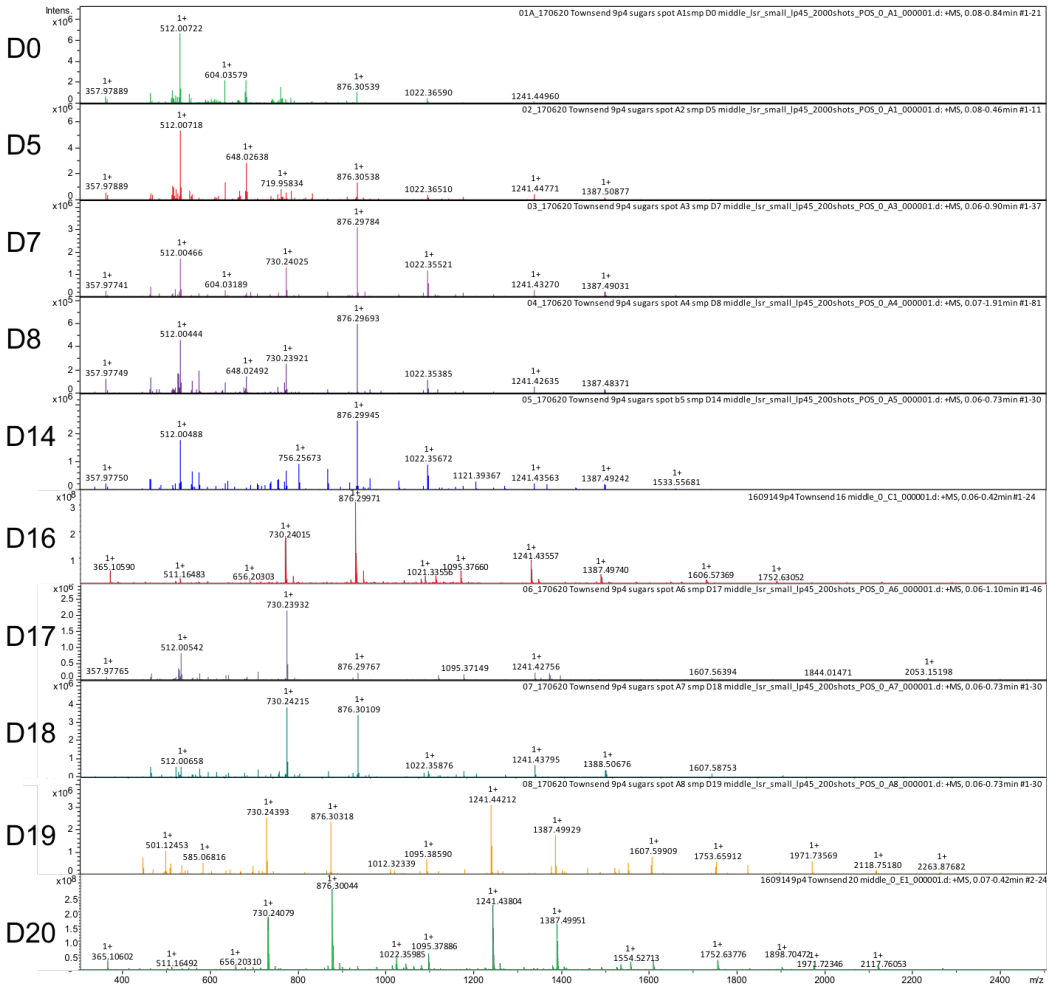


Figure A1. 1 Matrix-assisted laser desorption/ionization (MALDI) full size MS spectra for HMO mixtures isolated from Donors 0, 5, 7, 8, 14, 16, 17, 18, 19, and 20. Sample labels are listed to the left of each spectrum with a D# designation such that D0 corresponds to Donor 0, and so on. Samples were analyzed in positive ion mode on a 9.4T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (MS) (Bruker Solarix). Mass spectra were acquired in positive ion mode from *m/z* 300-2500. Sodium ion adducts of HMOs were detected with a mass accuracy of >2 ppm.

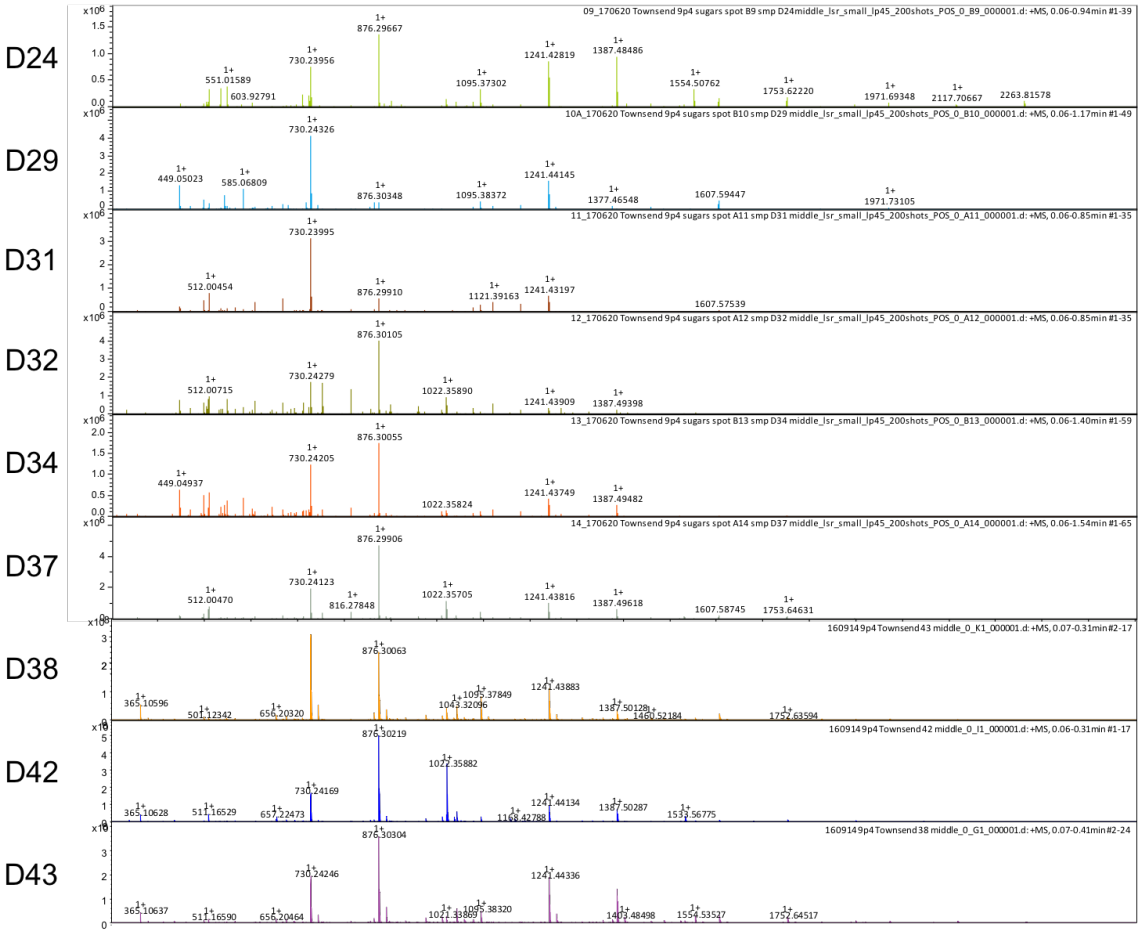


Figure A1. 2 Matrix-assisted laser desorption/ionization (MALDI) full size MS spectra for HMO mixtures isolated from Donors 24, 29, 31, 32, 34, 37, 38, 42, and 43. Sample labels are listed to the left of each spectrum with a D# designation such that D24 corresponds to Donor 24, and so on. Samples were analyzed in positive ion mode on a 9.4T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (MS) (Bruker Solarix). Mass spectra were acquired in positive ion mode from *m/z* 300-2500. Sodium ion adducts of HMOs were detected

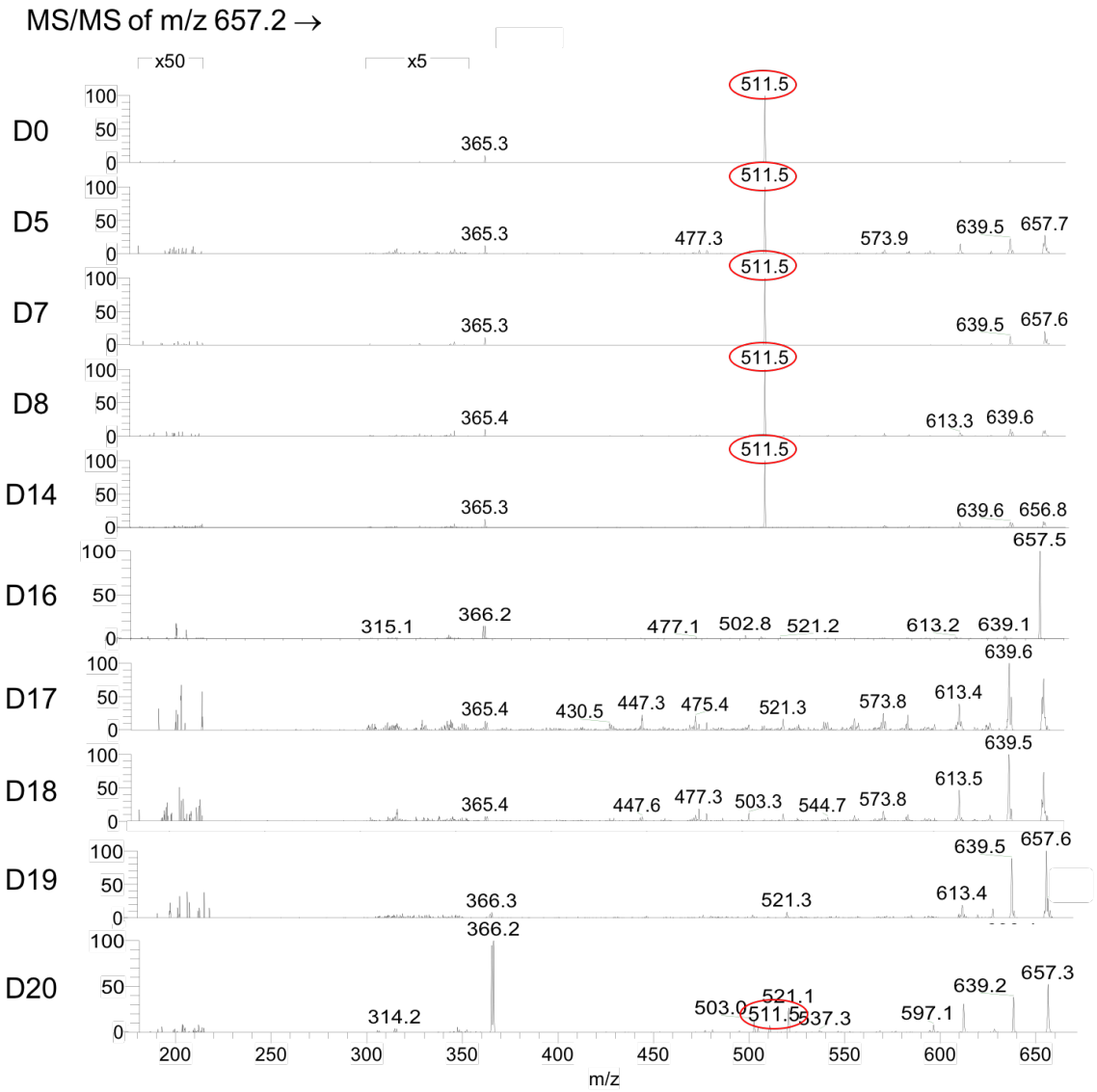


Figure A1. 3 Matrix-assisted laser desorption/ionization (MALDI) MS/MS spectra of selected *m/z* 657.2 ion for HMO mixtures isolated from Donors 0, 5, 7, 8, 14, 16, 17, 18, 19, and 20. Sample labels are listed to the left of each spectrum with a D# designation such that D0 corresponds to Donor 0, and so on. MS/MS analysis was performed with a linear ion trap mass spectrometer equipped with a MALDI source (LTQ XL, Thermo Scientific). Selected sodium adduct ions of interest were isolated with a 1 amu window and fragmented via CID using a collision energy of 35 eV. Ions circled in red are deterministic for Lewis blood group and secretor status assignment.

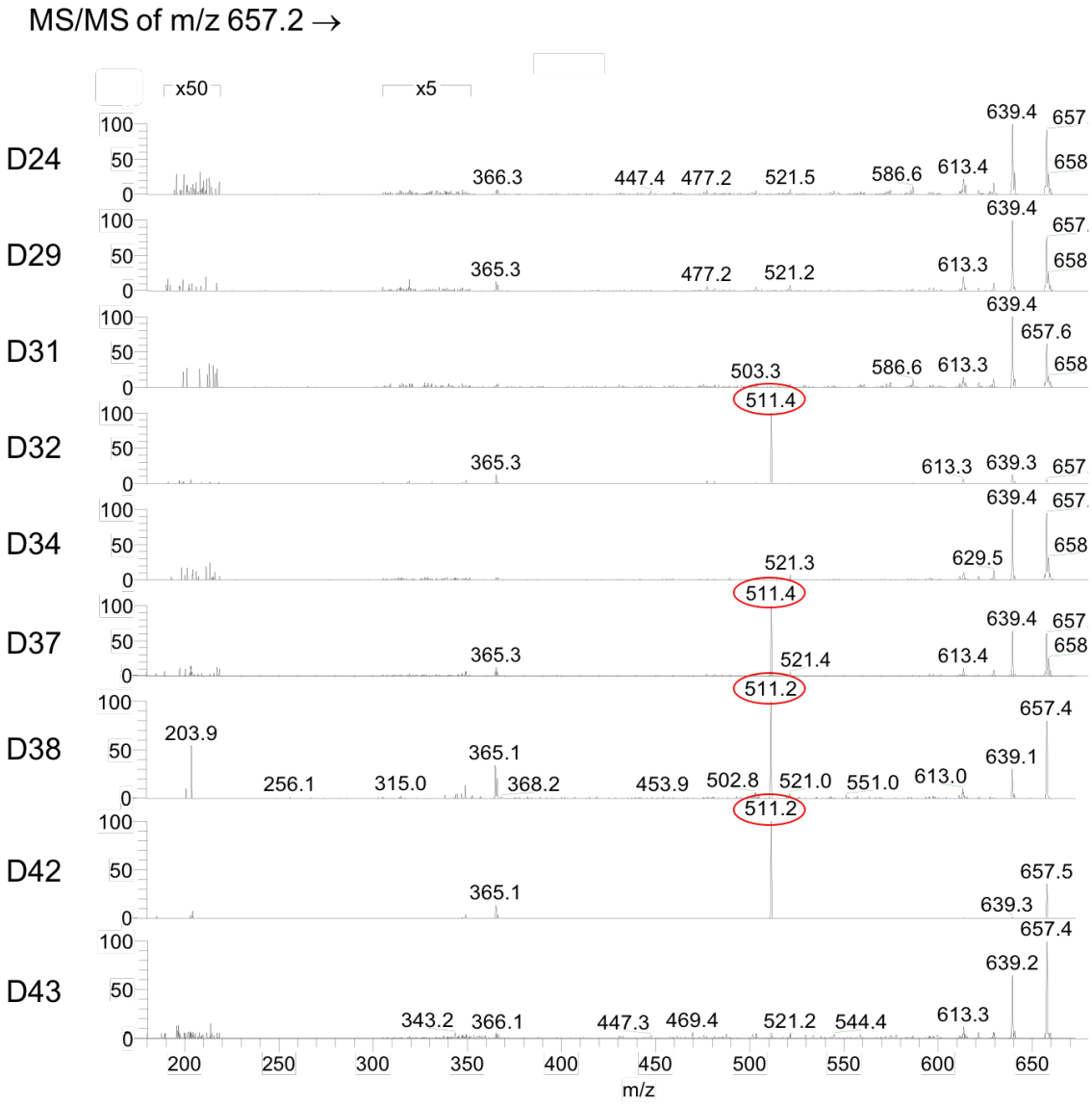


Figure A1. 4 Matrix-assisted laser desorption/ionization (MALDI) MS/MS spectra of selected m/z 657.2 ion for HMO mixtures isolated from Donors 24, 29, 31, 32, 34, 37, 38, 42, and 43. Sample labels are listed to the left of each spectrum with a D# designation such that D24 corresponds to Donor 24, and so on. MS/MS analysis was performed with a linear ion trap mass spectrometer equipped with a MALDI source (LTQ XL, Thermo Scientific). Selected sodium adduct ions of interest were isolated with a 1 amu window and fragmented via CID using a collision energy of 35 eV. Ions circled in red are deterministic for Lewis blood group and secretor status assignment.

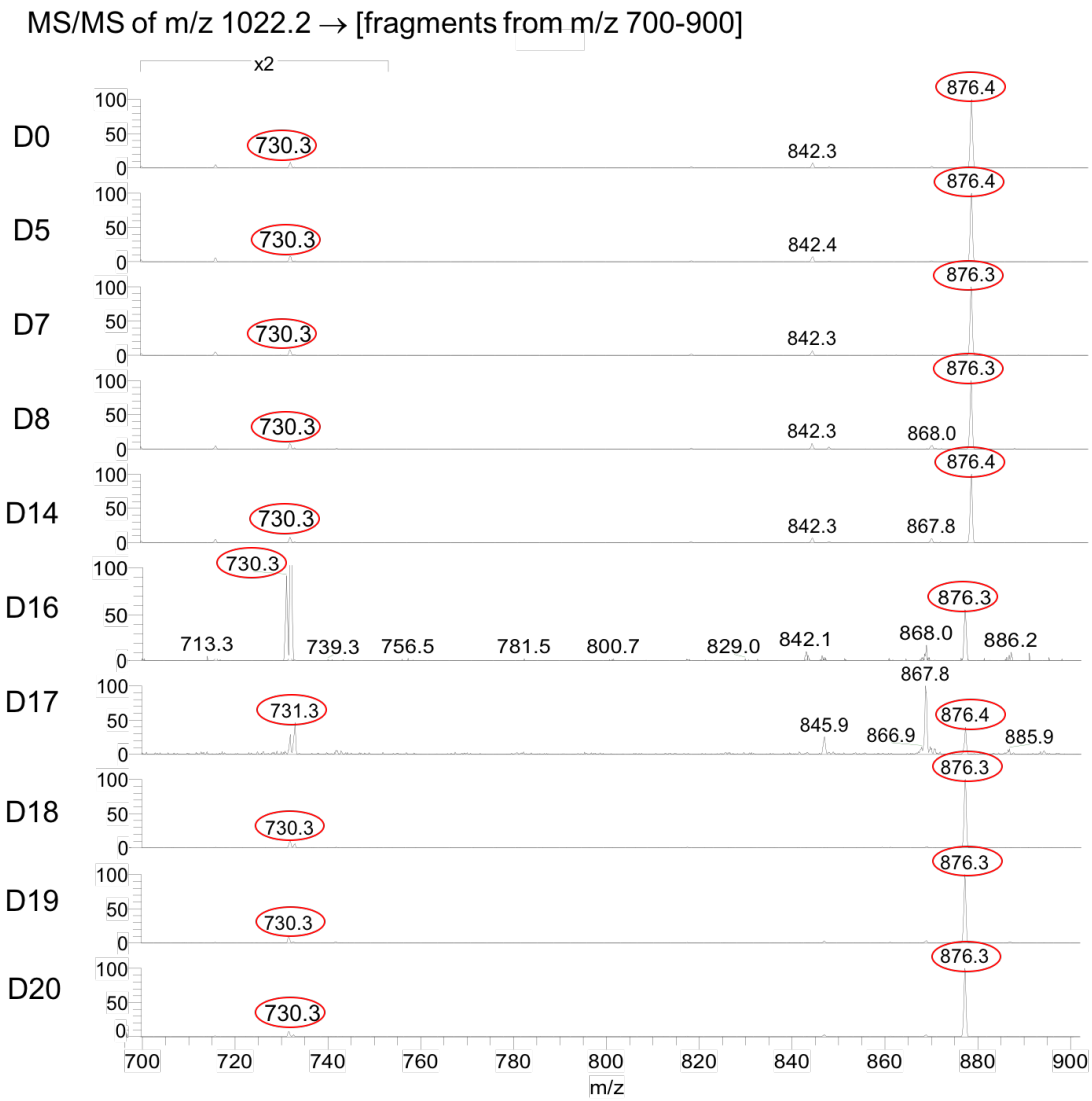


Figure A1. 5 Matrix-assisted laser desorption/ionization (MALDI) MS/MS spectra of selected m/z 1022.2 ion for HMO mixtures isolated from Donors 0, 5, 7, 8, 14, 16, 17, 18, 19, and 20. Sample labels are listed to the left of each spectrum with a D# designation such that D0 corresponds to Donor 0, and so on. MS/MS analysis was performed with a linear ion trap mass spectrometer equipped with a MALDI source (LTQ XL, Thermo Scientific). Selected sodium adduct ions of interest were isolated with a 1 amu window and fragmented via CID using a collision energy of 35 eV. Ions circled in red are deterministic for Lewis blood group and secretor status assignment.

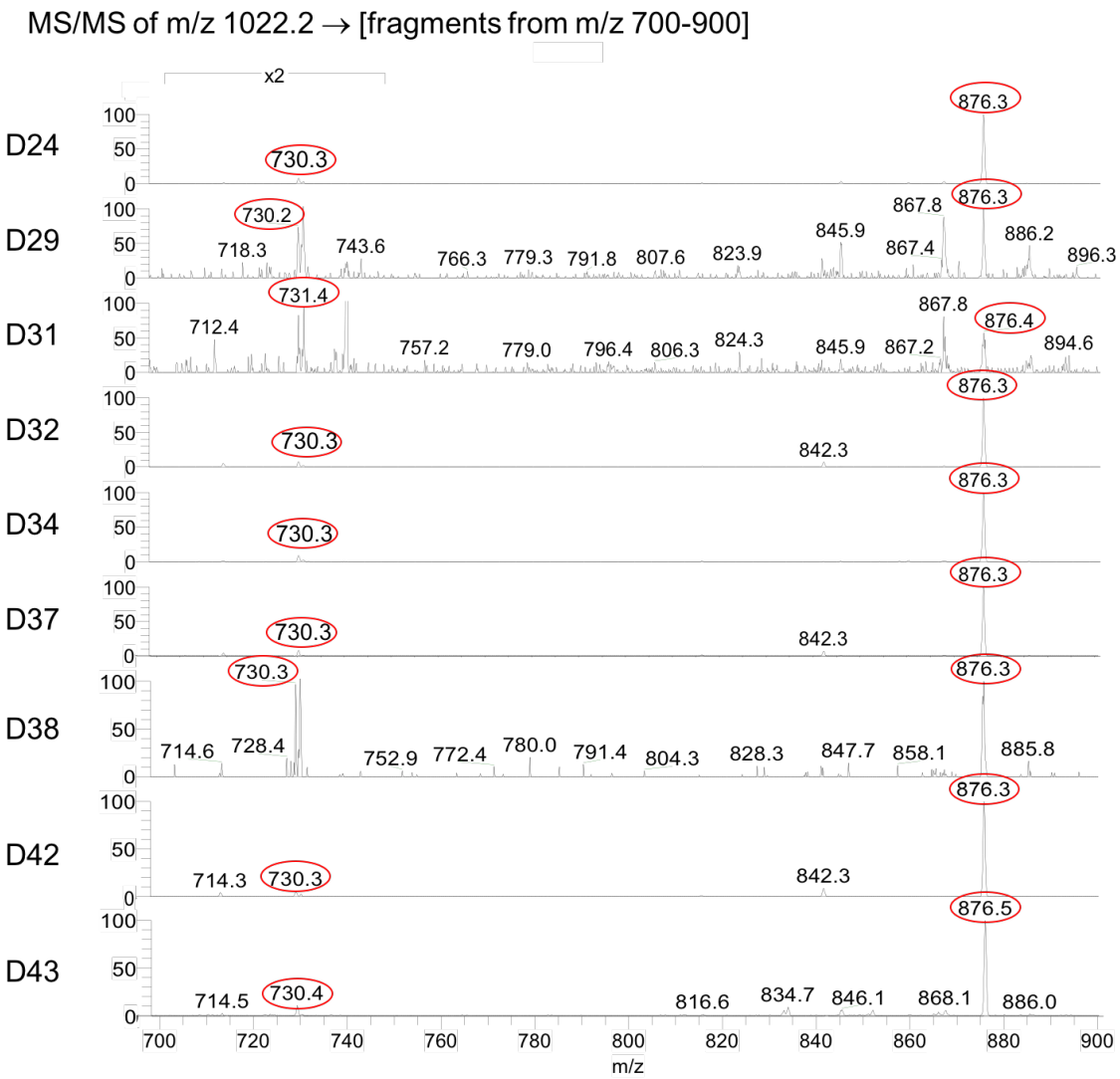


Figure A1. 6 Matrix-assisted laser desorption/ionization (MALDI) MS/MS spectra of selected m/z 1022.2 ion for HMO mixtures isolated from Donors 24, 29, 31, 32, 34, 37, 38, 42, and 43. Sample labels are listed to the left of each spectrum with a D# designation such that D24 corresponds to Donor 24, and so on. MS/MS analysis was performed with a linear ion trap mass spectrometer equipped with a MALDI source (LTQ XL, Thermo Scientific). Selected sodium adduct ions of interest were isolated with a 1 amu window and fragmented via CID using a collision energy of 35 eV. Ions circled in red are deterministic for Lewis blood group and secretor status assignment.

CHAPTER III

Human Milk Oligosaccharides Exhibit Antimicrobial and Anti-Biofilm Properties Against Group B *Streptococcus*

Adapted with permission from:
Ackerman, D. L.; Doster, R. S.; Weitkamp, J. H.; Aronoff, D. M.; Gaddy, J. A.; Townsend, S. D., Human Milk Oligosaccharides Exhibit Antimicrobial and Antibiofilm Properties against Group B *Streptococcus*, *ACS Infect. Dis.*, **2017**, *3* (8), pp 595–605
DOI: 10.1021/acsinfecdis.7b00064. Copyright 2017 American Chemical Society.
https://pubs.acs.org/doi/10.1021/acsinfecdis.7b00064

Ackerman, D. L.; Craft, K. M.; Doster, R. S.; Weitkamp, J. H.; Aronoff, D. M.; Gaddy, J. A.; Townsend, S. D., Antimicrobial and Antibiofilm Activity of Human Milk Oligosaccharides against *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Acinetobacter* baumannii, ACS *Infect. Dis.*, **2018**, *4* (3), pp 315–324 **DOI:** 10.1021/acsinfecdis.7b00183. Copyright 2017 American Chemical Society. https://pubs.acs.org/doi/10.1021/acsinfecdis.7b00183

Introduction

HMOs are a class of complex carbohydrates unique to human milk, and their bioactivity is well established. Both neutral and sialylated HMOs have previously been shown to modulate pathogen infection and proliferation. For instance, LNT inhibits binding of the parasite *Entamoeba histolytica* to human epithelial cells through the binding of Gal of LNT by a Gal/GalNAc specific lectin that otherwise mediates trophozoite attachment and invasion of host cells.[1] *Pneumococci* initiate infections by binding to ligands containing lacto-N-biose and N-acetyllactosamine.[2] LNnT and LSTc represent characteristic lung cell ligands and can prevent *Streptococcus pneumoniae* infections by serving as decoy receptors.[2] In *E. coli* infections, sialylated fractions of HMOs showed a notable capacity to prevent hemagglutination by binding colonization factor antigens.[3]

Sialylated HMOs also protect bladder cells from the cytotoxicity and inflammation caused by uropathogenic *E. coli* (UPEC) infections through disruption of UPEC intracellular signaling to prevent degradation of focal adhesion proteins which in turn activates signals leading to cell damage.[4] Fucosylated derivatives serve as decoy receptors for the adhesins and enterotoxins responsible for binding to host cells, and neutral, fucosylated derivatives have been shown to affect several pathogens including *E. coli*, *Campylobacter jejuni*, and norovirus.[5-12] [13, 14]

Group B *Streptococcus* (GBS) is a leading cause of neonatal infections. Recently the Bode and Le Doare research groups have separately reported the ability of HMOs to inhibit the proliferation of GBS *in vitro* and *in vivo*.[15-17]

A study conducted by Le Doare and coworkers analyzed the correlation between a mother's Lewis phenotype or secretor status and GBS colonization and transmission to the infant.[16] Proton nuclear magnetic resonance (^{1}H-NMR) was used to characterize milk samples based on the type and quantity of fucosylated HMOs present and to assign Lewis and secretor status to each donor. Chi-square analysis of maternal Lewis antigen status and maternal or infant colonization at delivery suggested that Lewis-positive mothers and their infants have lower rates of GBS colonization. However, secretor status did not prove to be statistically correlated to the incidences of GBS colonization. Further, they showed that increased concentrations of specific HMOs (3'-FL and LNDF-I) in maternal milk were correlated with decreased bacterial burden during colonization *in vivo*. An *in vitro* study showed that the presence of larger, branched fucosylated HMOs, such as LNDF-I, were associated with a reduction in GBS growth. In summary, they reported that fucosylated HMOs can inhibit GBS growth and colonization in a dose-dependent manner both *in vitro* and *in vivo*.

Bode and coworkers conducted a study to investigate the spectrum of antimicrobial activity exhibited by HMOs.[17] HMOs from several different donors were either pooled or pooled then separated into neutral and acidic fractions for analysis of activity. After growing several species of bacteria (Group B strep, *S. pyogenes* (Group A strep), *E. coli*, *P. aeruginosa*, and methicillin-resistant *Staphylococcus aureus*) in the presence of pooled HMOs, they concluded that HMOs exhibit narrow spectrum antibacterial activity against GBS and did not affect the growth of the other species. Further investigation showed that the antibacterial activity of HMOs extends across several serotypes of GBS and is dose dependent. Neutral HMO fractions were responsible for the antimicrobial activity, with LNT and LNFP-I showing the most significant activity as individual compounds. To probe the mechanism of action, a library of mutants was tested for their sensitivity to HMO antimicrobial activity. Interestingly, only one glycosyltransferase mutant (*gbs0738*) was able to proliferate in the presence of HMOs. While the exact role of this glycosyltransferase is under investigation, other glycosyltransferase mutants remained sensitive to HMO antibacterial activity.

On the basis of the established evidence that HMOs possess antimicrobial activity, we hypothesized milk oligosaccharides could modulate both the bacterial growth and biofilm production of GBS. Similar to other bacterial pathogens, GBS biofilm formation is an important virulence pathway known to provide increased resistance to antimicrobial agents as well as host defenses.[18] Previous research has demonstrated the importance of GBS CPS biosynthesis in mediating biofilm formation, supporting our hypothesis that oligosaccharides could influence biofilm establishment.[19, 20] Additionally, GBS CPS from type Ib and II are similar in structure to certain HMOs (Figure 3.1).[21, 22] A study by Pritchard and co-workers showed mouse antibodies for GBS CPS bind to HMOs, further suggesting that their structural similarity enable HMOs to

mimic or influence CPS activity.[21]

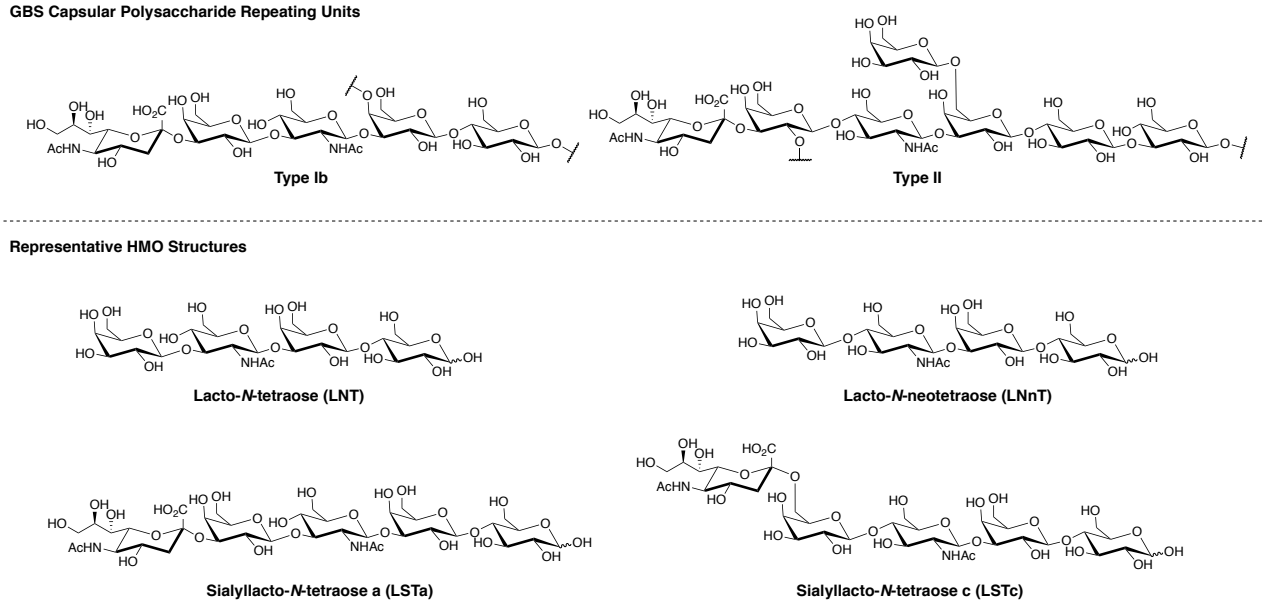


Figure 3. 1 *S. agalactiae* Capsular Polysaccharide Repeat Units Share Structural Elements of Human Milk Oligosaccharides.

In this chapter, we detail two related studies that report the effect of oligosaccharide isolates from donor human milk samples on the growth, biofilm formation, and biofilm architecture of GBS. In an initial study, the antimicrobial properties as well as the effect on biofilm formation and biofilm architecture were evaluated for oligosaccharides from five donor human milk samples. Using one GBS strain, this study included an investigation into both the qualitative and quantitative effects on biofilm by examining the structures and components of the biofilms formed in the presence of these HMO isolates. A second study expanded on our initial findings to report a broader evaluation of the antimicrobial and anti-biofilm activity by including HMO isolates from fourteen additional donor milk samples as well as three GBS stains of unique serotypes.

Initial Study

We sought to understand the impact of HMOs on the growth and viability of GBS. Since we were interested in the effects of HMOs on both growth and biofilm formation, assays were conducted in two growth conditions: Todd Hewitt Broth (THB) and THB supplemented with 1% glucose. Glucose supplementation is thought to increase biofilm formation through acidification of the media, resulting in the upregulation of biofilm formation.[18,23]

HMOs were isolated from five donors, and Lewis blood groups were assigned as described in Chapter 2 (Table 2.5, donors 16, 20, 38, 42, and 43). Each donor milk sample was received pre-labeled with a donor number that was kept associated with each sample of isolated HMOs. Carbohydrate concentrations of roughly 5 mg/mL were used to represent a low physiological concentration of HMOs, which can range from 5-20 g/L.[23, 24] During the carbohydrate isolation process, precautions were not taken to remove lactose, which is highly abundant in human milk, and a concentration of 5 g/L represents a combination of lactose and HMOs. GBS strain CNCTC 10/84, which is categorized as serotype V, was used for this study.

HMOs Modulate Growth and Viability of S. agalactiae

First, GBS cells were grown in THB in the presence or absence of HMOs from each donor breast milk sample. Culture turbidity was measured spectrophotometrically at 600 nm (OD_{600}, a common wavelength to measure bacterial culture turbidity)[25] as a measurement of bacterial growth, and samples were serially diluted and plated on blood agar plates to confirm bacterial cell viability. HMOs from donor 43 demonstrated marked antimicrobial activity against GBS compared with media alone (Figure 3.2, Figure A2.1), resulting in approximately 90% growth

inhibition over 24 h. Additionally, HMOs from donor 38 significantly inhibited GBS growth for the first 8 h of culture (Figure 3.2, Figure A2.1), with percent inhibition holding near 50% between 4− 6 h and dropping to 27% at 7 h. HMOs from donors 16, 20, and 42 showed no significant effect on GBS growth in THB.

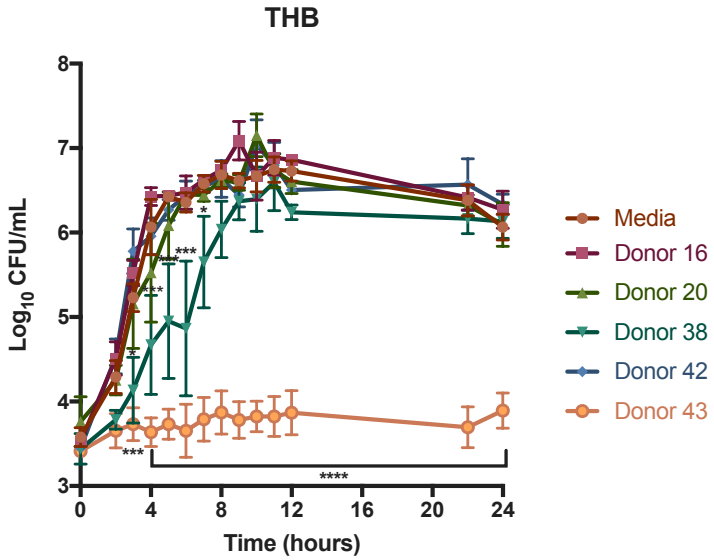


Figure 3. 2 Effect of HMOs isolated from individual milk samples on GBS CNCTC 10/84 proliferation in Todd Hewitt Broth. Enumeration of CFU was performed at 0, 2−12, 22, and 24 h. The mean CFU/mL was calculated for each time point and is indicated by the respective symbols. Data displayed represents the mean CFU/mL ± SEM of 3 biological replicates, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by 2-way ANOVA with posthoc Dunnett's mutiple comparison test, with all donor samples compared to the GBS growth in media alone.

Next, GBS cells were grown in THB containing 1% glucose in the presence or absence of HMO samples. Growth was measured as described previously. Similar to growth in THB, HMOs from donor 43 demonstrated growth inhibition as high as 80% against GBS compared with the control (Figure 3.3, A2.2). However, growth increases after 22 h, suggesting that the HMOs from donor 43 may be acting as a bacteriostatic agent. HMOs from all other donors (16, 20, 38 and 42) showed no significant effect on GBS growth in THB supplemented with 1% glucose.

The half maximal inhibitory concentrations (IC_{50}) in THB were determined for HMOs

from donors 38 and 43, which inhibited growth. HMOs from donor 38 had an IC_{50} of 23.2 mg/mL, which is just above the range of typical physiological concentrations of HMOs in human milk (5-20 mg/mL). Correlating to its greater antimicrobial activity, HMOs from donor 43 had an IC_{50} of 2.44 mg/mL, which is below typical physiological concentrations.

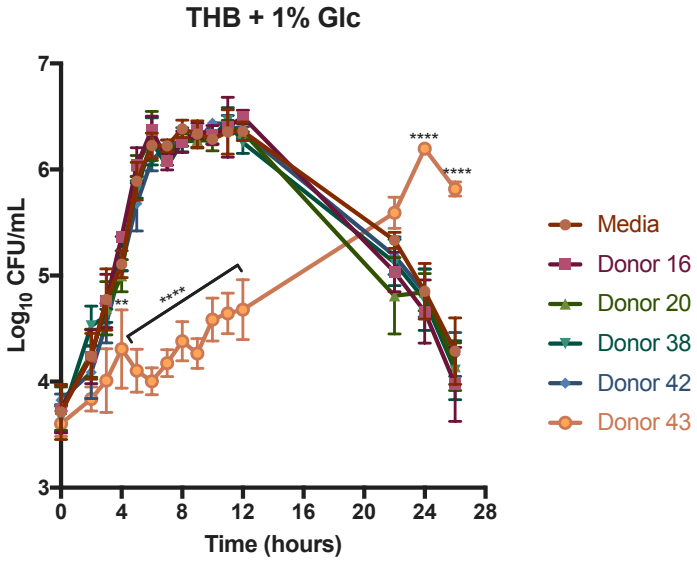


Figure 3. 3 Effect of HMOs isolated from individual milk samples on GBS CNCTC 10/84 proliferation in Todd Hewitt Broth supplemented with 1% glucose. Enumeration of CFU was performed at 0, 2−12, 22, and 24 h. The mean CFU/mL was calculated for each time point and is indicated by the respective symbols. Data displayed represents the mean CFU/mL ± SEM of 3 biological replicates, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by 2-way ANOVA with posthoc Dunnett's multiple comparison test, with all donor samples compared to the GBS growth in media alone.

Evaluation of HMO Effect on GBS Biofilm Formation

In order to evaluate biofilm formation, a plate-based biofilm assay was used that measures bacterial growth as well as biofilm production by crystal violet (CV) staining. After growth media is aspirated, CV is used to stain the entire biofilm including cells and extracellular polymeric matrix.[26] After staining and rinsing, CV that remains is solubilized and the amount of CV is measured spectrophotometrically using OD_{560}, a wavelength used for CV absorbance. Biofilm production is expressed as a ratio of the biofilm produced (OD_{560}) to the number of bacterial cells

present (OD_{600}, biomass). This allows for a quantitative measurement of the amount of biofilm produced relative to growth (biomass turbidiy measurement).

GBS biofilm production was largely unaffected by the presence of HMOs in the growth media (Figure 3.4A). HMOs from donor 43 significantly increased the biofilm/biomass ratio of cells grown in THB by over 200% ($p = 0.0008$ by one-way ANOVA with posthoc Dunnett's multiple comparison test) but had no effect on biofilm/biomass ratio when the media was supplemented with glucose. This result is likely due to the inhibition of cell growth (as shown in Figure 3.2), which is in contrast to the restored growth observed after 22 h in THB with 1% glucose (Figure 3.3). When the biomass is significantly decreased (as is the case for donor 43), the ratio of biofilm to biomass becomes significantly higher because biofilm production is measured as a ratio of biofilm to biomass.

When GBS cells were grown in media supplemented with glucose, HMOs from donor 38 diminished the biofilm/biomass ratio by 17% compared to the control grown in media alone ($p = 0.0018$ by one-way ANOVA with posthoc Dunnett's multiple comparison test) (Figure 3.4B). A comparison of the relative biofilm amounts produced in both THB and THB supplemented with 1% glucose are shown in Appendix 2 (Figure A2.3).

Microscopic Evaluation of Biofilms Grown in the Presence of HMOs

In addition to biofilm quantification, we evaluated if incubation in the presence of HMOs could induce structural changes to GBS biofilms. Biofilms were grown in media supplemented with 1% glucose to enhance biofilm formation.

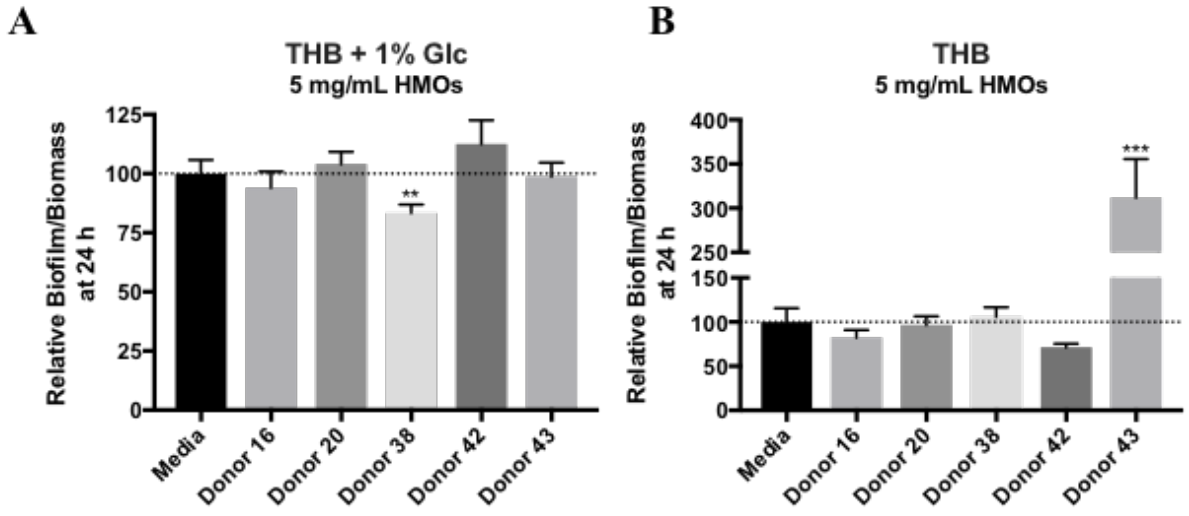


Figure 3. 4 HMOs at biologically relevant breast milk concentrations induce changes in biofilm formation of GBS cultures. The total biofilm to biomass ratio after 24 h of growth was compared for (A) THB medium alone. Data represented as the mean biofilm/biomass ratio ± SEM of 5 separate experiments, each with 3 technical replicates. *** represents $p = 0.0008$ by one-way ANOVA, F = 23.35 with posthoc Dunnet's multiple comparison test comparing each HMO group against the control sample without HMOs. (B) THB medium supplemented with 1% glucose. Data are expressed as the percent mean biofilm/biomass ratio of control ± SEM of 5 separate experiments, each with 3 technical replicates. ** represents $p = 0.0018$ by oneway ANOVA, F = 3.449 with posthoc Dunnet's multiple comparison, compared to media alone.

High resolution scanning electron microscopy (SEM) was used to analyze and evaluate changes in biofilm architecture and size. SEM can reach a much higher resolution and magnification than light microscopy and uses a beam of electrons repeatedly scanned across a sample's surface to create an image and to gain information about the surface features. SEM analysis of GBS biofilms allows for comparison of the packing structure of the biofilms as well as formation of nutrient channels for samples grown in the presence and absence of HMOs.

Compared to media alone, GBS cells incubated with HMOs from donor 43 demonstrated less diffuse biofilms and smaller biofilm mushroom structures (Figure 3.5). Additionally, GBS biofilms grown in the presence of HMOs from donors 16 and 38 had less prominent nutrient channels compared to GBS biofilms grown in the presence of HMOs from donors 20 and 42. This

observation aligns with the breast milk donor categorization of Lewis blood groups by MALDI profiling, suggesting that HMOs associated with certain Lewis blood groups may lead to alterations in GBS biofilm structure.

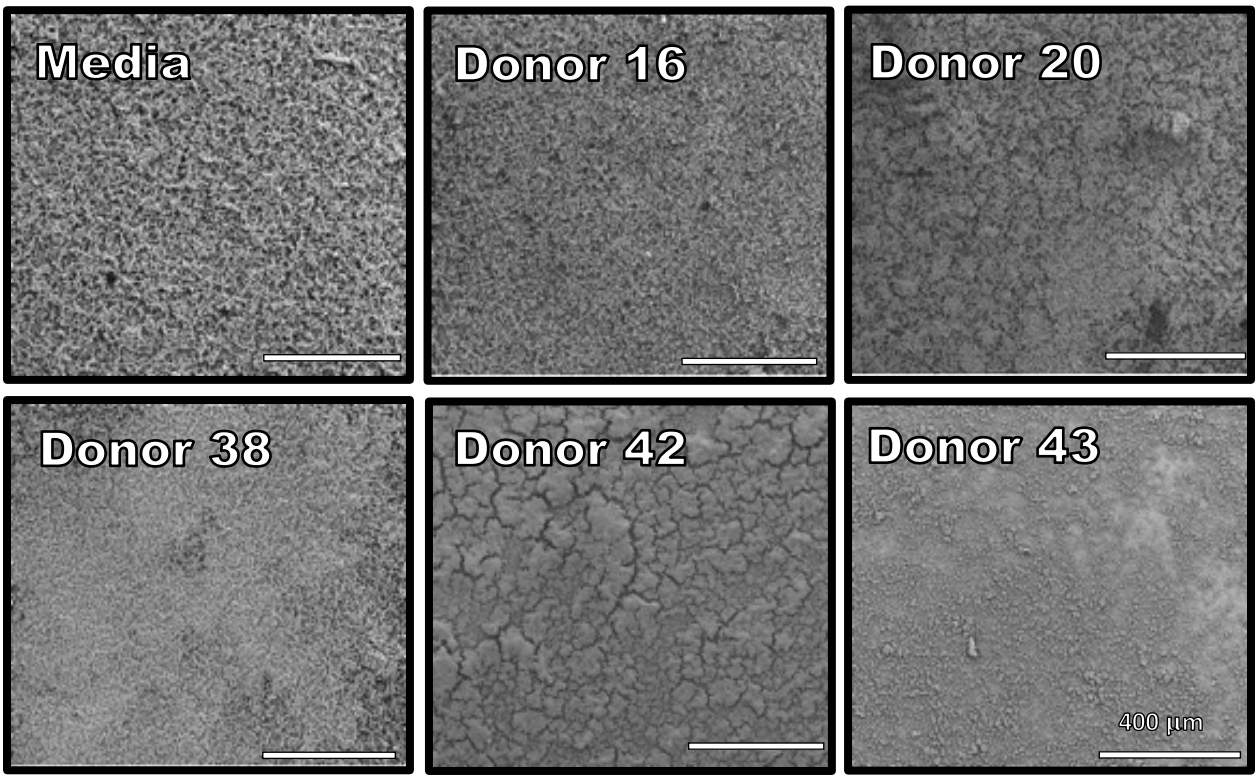


Figure 3. 5 Scanning electron micrographs of biofilm formation after 24 h. GBS CNCTC 10/84 cells were grown in THB + 1% glucose supplemented with individual donor samples for 24 h at 37 °C. Images are shown at 250x magnification.

We then examined these biofilms at higher magnification to visualize finer details in biofilm structure. While most donor samples had little effect on the cellular organization of the biofilm, SEM analysis at high magnification revealed that samples grown with HMOs from donor 43 caused changes in GBS chaining morphology. GBS strain CNCTC 10/84 phenotypically forms long chains of bacteria within the biofilm structure. However, HMOs from donor 43 induced a

truncated chain phenotype compared to the control sample resulting in a denser packing morphology within the biofilm (Figure 3.6).

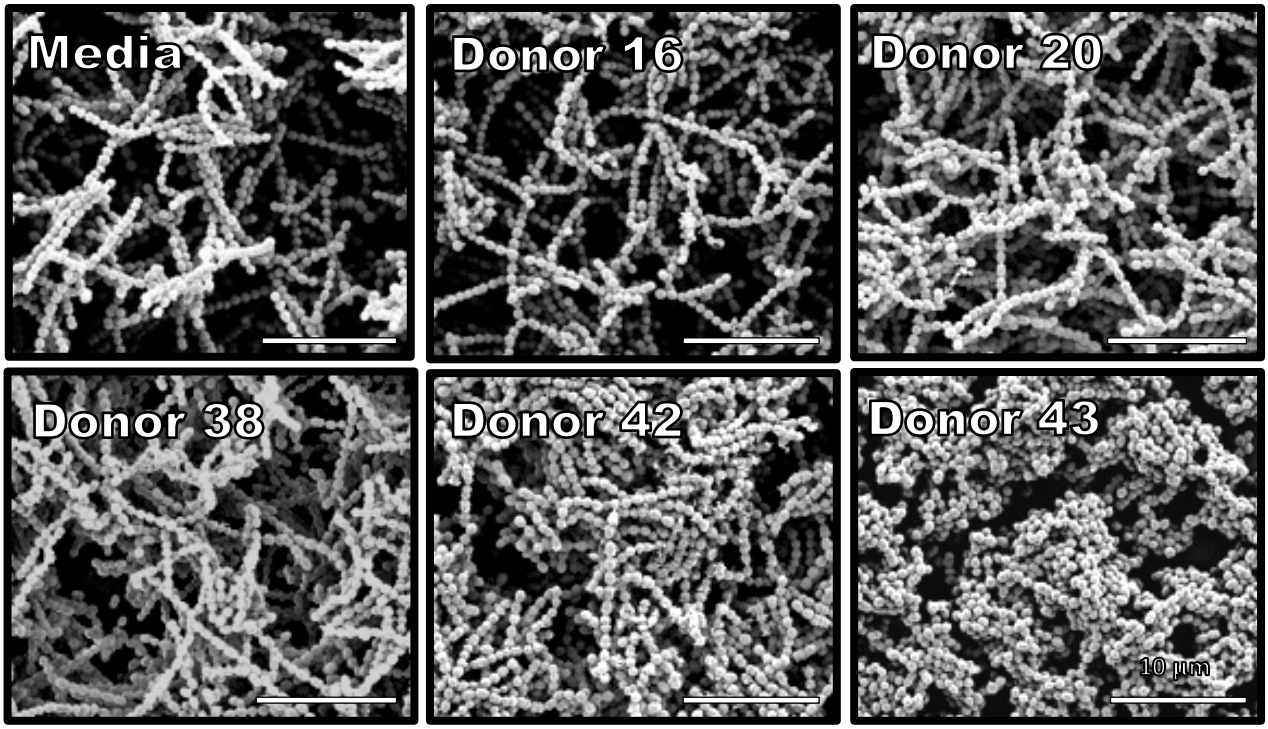


Figure 3. 6 Scanning electron micrographs of biofilm formation after 24 h. GBS CNCTC 10/84 cells were grown in THB + 1% glucose supplemented with HMOs from individual donor samples for 24 h at 37 °C. Images are shown at 1000x magnification.

Structural and compositional aspects of GBS 10/84 biofilms were analyzed using confocal laser scanning microscopy (CLSM). CLSM is a form of light microscopy that uses a narrow beam of light to change the depth of focus so that images can be taken in thin optical sections at different levels in a third dimension (z-plane). These images can be displayed to show the thickness in the z-dimension of an x-y snapshot (called a z-stack), or they can be stacked to generate a 3-D image. When CLSM is paired with the use of various cellular stains, different aspects of a specimen can be analyzed. For instance, live cells, dead cells, and carbohydrates can be uniquely stained and

imaged separately for individual analysis or overlay comparisons.

Biofilms grown in the presence of HMOs from donor 38 showed a decrease in thickness of the biofilm relative to biofilms grown in media alone as seen by a comparison of the x- and y-axis views of Figure 3.7. This is in agreement with the decrease in biofilm production seen in the plate-based assay (Figure 3.3B). Additionally, a comparison of the first and last z-stack images shows a greater carbohydrate (blue) content at the apical surface of the biofilm relative to the base, which is mostly composed of dead (red) and live (green) cells (Figure 3.8).

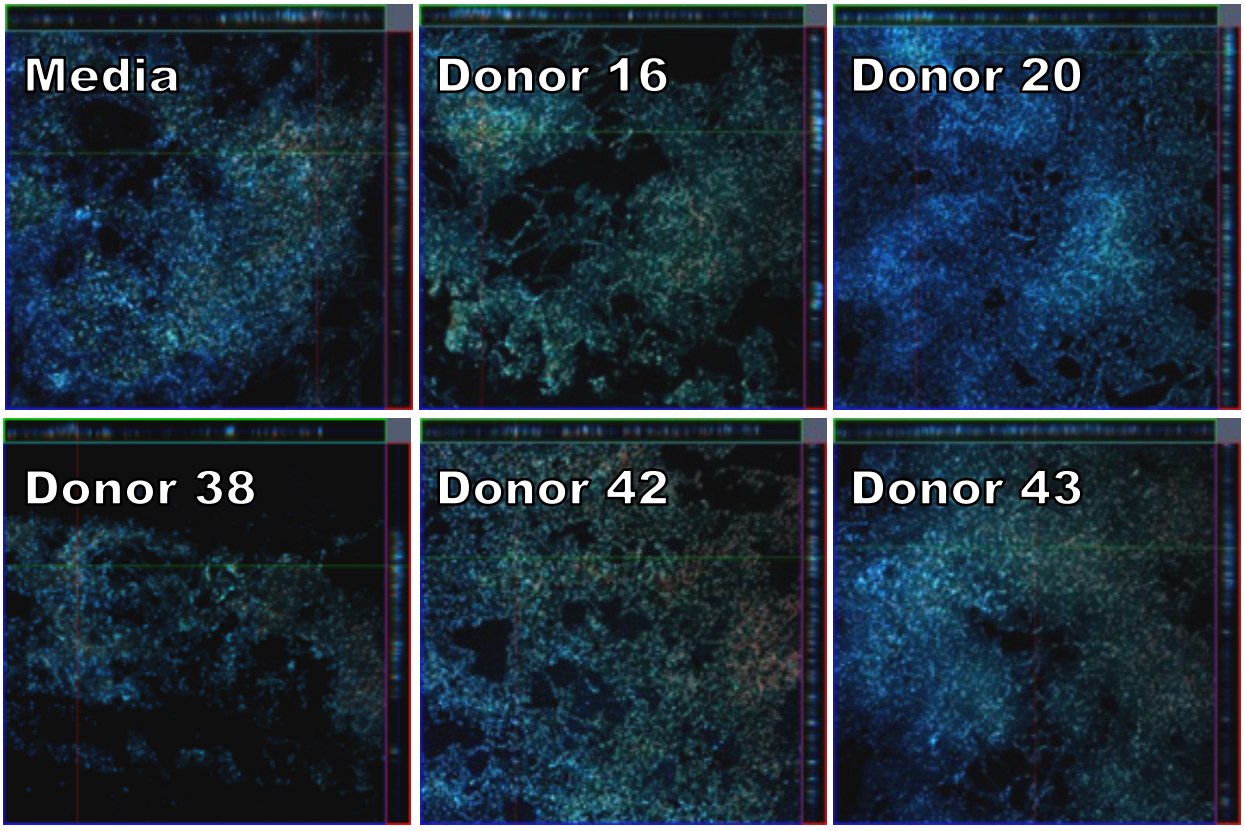


Figure 3. 7 CLSM micrographs comparing biofilm formation of GBS CNCTC 10/84 grown in THB supplemented with 1% glucose or THB supplemented with 1% glucose and HMOs isolated from milk donors. Bacteria were grown under static conditions at 37 °C for 24 h on glass coverslips. Biofilms were stained immediately prior to analysis with SYTO 9 (green, live bacterial cells), propidium iodide (red, dead bacterial cells), and Calcofluor White (blue, carbohydrates) and imaged at 600Å~ magnification. Images shown represent a z-stack series of images

of the three stains where the larger panel is a "bird's eye" view of the biofilms and the right and upper panels are side views of the x- and y-axis sections, respectively.

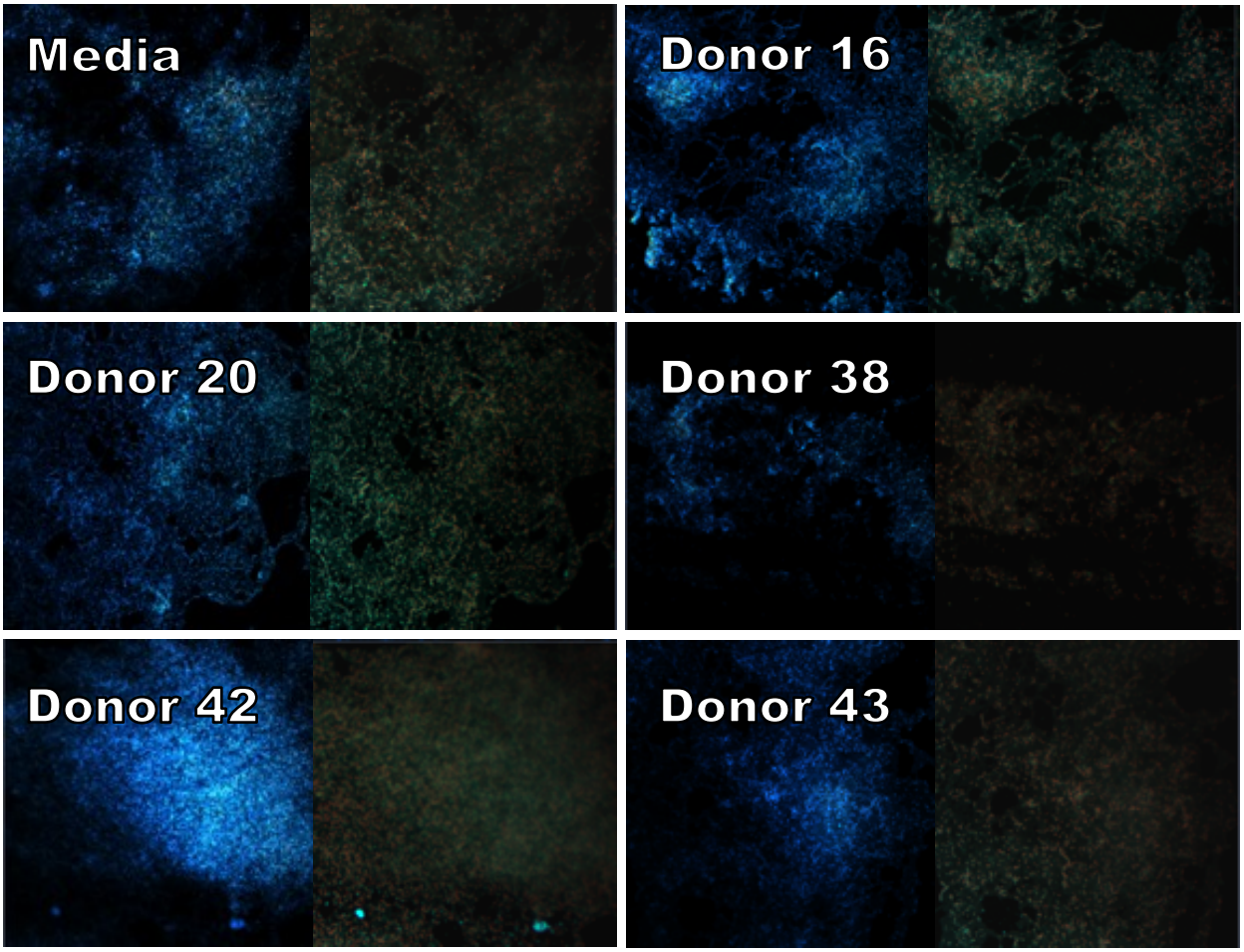


Figure 3. 8 CLSM micrographs comparing apical and base sections of GBS CNCTC 10/84 biofilms grown in THB supplemented with 1% glucose or THB supplemented with 1% glucose and HMOs isolated from milk donors. Bacteria were grown under static conditions at 37 °C for 24 h on glass coverslips. Images shown represent the apical surface (left image) and base of the biofilm (right image) from a z-stack series. Biofilms were stained with SYTO 9 (green, live bacterial cells), propidium iodide (red, dead bacterial cells), and Calcofluor White (blue, carbohydrates) and imaged at 600Å~ magnification.

Conclusions to initial study

We have demonstrated that HMOs isolated from distinct donors exhibit antimicrobial properties against GBS. Moreover, we have shown that HMOs also disrupt the formation and structure of biofilms produced by this pathogen.

Given the discovery of the modulatory effects of HMOs against GBS, it is peculiar to note the diverse activity displayed by glycans from each donor (Table 3.1). HMOs from Donor 43 significantly inhibited the growth of GBS in both THB (Figure 3.2) and THB supplemented with 1% glucose (Figure 3.3) and changed the morphology of the biofilm (Figure 3.6). HMOs from donor 38 significantly inhibited GBS growth in THB for the first 8 h of a 24 h growth period (Figure 3.1) and significantly decreased *in vitro* biofilm production as measured by the biofilm/biomass ratio (Figure 3.4B). Additionally, HMOs from donors 38 and 43 affected the visual nutrient channel formation of GBS biofilms as shown by SEM (Figure 3.5). HMOs from donors 16, 20, and 42 showed no significant antibacterial or anti-biofilm effects.

Table 3. 1 Summarization of the effect of HMOs on GBS

Donor	Lewis Blood Group	Growth Change from Control (%)[a]		Biofilm Change from Control (%)	
		THB	THB+1%Glc	THB	THB+1%Glc
16	**a-b-**	-7 ± 7	+1 ± 5	-18 ± 9	-6 ± 7
20	**a-b+**	+4 ± 7	-5 ± 5	-3 ± 10	+4 ± 5
38	**a-b-**	-14 ± 5	0 ± 8	+6 ± 11	**-17 ± 3**
42	**a-b+**	-6 ± 9	-10 ± 6	-29 ± 4	+13 ± 10
43	**a+b-**	**-80 ± 11**	**-30 ± 4**	+212 ± 44	-1 ± 6

[a]Growth change from control for t = 24 h (THB) and t = 22 h (THB+1%Glc) of growth during growth curve assays using OD_{600} values.

This study of the antimicrobial and anti-biofilm properties of HMOs from various Lewis blood groups suggested that milk of mothers might differentially influence infant health in relationship to GBS. This conclusion follows from the study by Le Doare and coworkers that showed a correlation between Lewis status of the mother and incidence of GBS infections in both the mother and infant.[16] Other studies have shown compounds associated with secretor milks,

specifically α-1,2-fucosylated HMOs, reduce incidence of diarrhea caused by infections with *E. Coli* and *C. Jejuni*.[7, 8, 13, 27, 28] We hypothesized that there could be a relationship between the Lewis blood group of the mother and the antimicrobial or anti-biofilm activity of the HMOs in her milk. Testing this hypothesis was the goal of the expanded study described below. Additionally, increasing the number of individual donor milk samples would provide information about the extent of variability in effects of HMOs on GBS pathogenesis.

Expanded Study

After confirming the antibacterial activity and establishing anti-biofilm activity of HMOs against GBS, we sought to expand the number of HMO samples studied in order to investigate a potential relationship between Lewis blood group and biological activity. Prior to bacterial assays, Lewis blood groups for the fourteen additional donors were assigned using the high throughput mass fingerprinting technique developed by Kunz and co-workers (Table 2.4, donors 0, 5, 7, 8, 14, 17, 18, 19, 24, 29, 31, 32, 34, and 37).[29]

Next, we tested the hypothesis that HMOs act as antimicrobial and anti-biofilm agents across several strains of GBS. Thus, three strains of *S. agalactiae* of varying serotypes were used: CNCTC 10/84, GB590, and GB2. CNCTC 10/84 is a Type V strain, whereas GB590 is a Type III, and GB2 is a Type Ia strain. These three serotypes account for over 85% of invasive infant GBS disease cases worldwide (Figure 1.7).[30] Antimicrobial and anti-biofilm activities were evaluated at 24 h in both THB and THB supplemented with 1% glucose using carbohydrate concentrations of 5 mg/mL. As before, no precautions were taken to remove lactose, so carbohydrate concentrations included a mixture of lactose and HMOs.

HMOs Modulate Growth and Viability of S. agalactiae

To determine the antimicrobial activity, we used the same plate-based assay described above, which allows for spectrophotometric quantification of both bacterial growth and biofilm production. We compared the biomass of bacteria grown in the presence of HMOs to that of bacteria grown in media alone for both growth conditions (THB and THB supplemented with 1% glucose). Several HMO samples were found to significantly inhibit bacterial growth for the three GBS strains in both growth conditions ($p \leq 0.05$ by one-way ANOVA with posthoc Dunnett's multiple comparison test) (Table 3.2, Figure A2.4, A2.5, A2.6). The results are presented as the average percent deviation from the control ± standard error of the mean (SEM) of three independent experiments each with three technical replicates where negative numbers represent inhibition of bacterial growth and positive numbers represent an increase in bacterial growth.

In THB, HMOs from donors 5, 8, and 29 inhibited the growth of GBS at 24 hours across all three strains tested (CNCTC 10/84, GB590, GB2). HMOs from donor 8 had the greatest impact on growth, exhibiting an 75-89% decrease relative to the control, but only exhibited growth inhibition in THB. HMOs from donor 29 had the next greatest reduction of growth in THB, ranging from 15-42% relative to the control. HMOs from donor 5 were able to inhibit growth compared to the control by 22-31%. Additionally, for GBS strain CNCTC 10/84, HMOs from donors 18, 24, and 32 significantly inhibited the growth in THB, with reductions of 13, 11, and 14%, respectively.

While the ability to inhibit growth was diminished in THB supplemented with glucose, HMOs from donors 5, 29, 32, and 37 still exhibited significant growth inhibition relative to control in GBS strain CNCTC 10/84 (Table 3.2, Figure A2.7, A2.8, A2.9) with observed inhibitions of

12, 17, 16, and 17%, respectively. The growth of the other two strains (GB590 and GB2) was either unaffected or increased in the presence of HMOs (Table 3.2, A2.10, A2.11, A2.12).

Notably, HMOs from donor 8 reduced growth by an average of over 70% for all GBS strains when grown in glucose free media. In contrast, when GBS was grown in media supplemented with 1% glucose, donor 8 HMOs decreased growth by less than 10% for all strains. Overall, greater HMO antimicrobial activity was seen in THB than THB supplemented with 1% glucose. This is likely due to the ability of GBS to proliferate at an accelerated rate in glucose, allowing the bacteria to overcome the antimicrobial effects of the HMOs.[31]

Evaluation of HMO Effect on GBS Biofilm Formation

We assessed changes in biofilm productionby comparing biofilm/biomass ratios of bacteria grown in the presence of HMOs to those grown in media alone. HMOs from each donor sample significantly reduced biofilm formation in at least one GBS strain (Table 3.3). It is important to note that in order to determine significant reductions in biofilm production when GBS was grown in THB, the results from Donor 8 were omitted from analysis of all strains. Results from Donor 8 were confirmed to be outliers by both ROUT (Q = 1%) and Grubbs (a = 0.05) outlier tests. It is likely the exceptionally high biofilm/biomass ratios seen for Donor 8 HMOs are attributable to the extreme reduction in bacterial growth when bacteria were grown in THB (Table 3.2, Figure A2.4, A2.5, A2.6). When in THB, HMOs from this donor caused at least a 75% reduction in biomass compared to the control across the three strains. With the less dramatic antimicrobial activity of HMOs from Donor 8 in THB supplemented with 1% glucose, the biofilm/biomass ratios return to more reasonable values in this growth medium.

Table 3. 2 Antimicrobial Activity of HMOs against Three Strains of Group B *Streptococcus*[a]

		Biomass Change from Control (%)					
		CNCTC 10/84		**GB590**		**GB2**	
Donor	**Lewis Blood Group**	**THB**	**THB +1%Glc**	**THB**	**THB +1%Glc**	**THB**	**THB +1%Glc**
0	a-b+	-4 ± 2	+11 ± 2	+14 ± 3	+11 ± 3	+5 ± 2	+9 ± 2
5	a-b+	**-26 ± 1**	**-12 ± 2**	**-31 ± 6**	-9 ± 2	**-22 ± 1**	-5 ± 1
7	a-b+	-3 ± 1	+13 ± 4	+6 ± 3	+8 ± 2	-1 ± 2	-3 ± 2
8	a-b+	**-80 ± 6**	-5 ± 2	**-75 ± 9**	-8 ± 5	**-89 ± 4**	-6 ± 2
14	a-b+	+3 ± 1	+43 ± 1	+8 ± 4	+50 ± 2	+14 ± 2	+57 ± 1
19	a-b+	-8 ± 2	+7 ± 3	+13 ± 1	+28 ± 2	+1 ± 2	+14 ± 3
24	a-b+	**-11 ± 3**	+8 ± 1	+11 ± 3	+20 ± 2	+9 ± 3	-3 ± 1
32	a-b+	**-14 ± 1**	**-16 ± 3**	+10 ± 2	+15 ± 3	+14 ± 2	+6 ± 2
34	a-b+	+2 ± 1	-2 ± 3	+21 ± 3	+25 ± 4	+15 ± 2	+19 ± 5
37	a-b+	-1 ± 2	**-17 ± 3**	+23 ± 3	+24 ± 3	0 ± 2	+19 ± 3
17	a+b-	-2 ± 1	+4 ± 4	+7 ± 2	+17 ± 3	+7 ± 2	+17 ± 4
18	a+b-	**-13 ± 3**	+11 ± 1	-11 ± 3	+14 ± 2	-1 ± 2	-6 ± 2
29	a+b-	**-42 ± 1**	**-17 ± 2**	**-35 ± 11**	-22 ± 6	**-15 ± 1**	-6 ± 1
31	a+b-	-6 ± 2	+18 ± 2	+3 ± 2	+33 ± 4	+7 ± 2	+24 ± 3

[a]Significant growth inhibition ($p \leq 0.05$, one-way ANOVA) compared to control is boldfaced)

For GBS strain CNCTC 10/84, HMOs from donors 0, 5, 14, 17, 19, 24, 29, 32, and 37 significantly inhibited biofilm formation in THB, with inhibitions ranging from 53-80% (Table 3.3, Figure A2.13). In THB supplemented with 1% glucose, HMOs from donors 7, 14, 18, 24, and 31 showed significant anti-biofilm activity by reduction of biofilm production by 36-81% relative

to the control (Table 3.3, Figure A2.14).

GB590 was less susceptible to inhibition of biofilm production caused by HMOs. In THB, no HMOs were able to reduce the biofilm production significantly (Table 3.3, Figure A2.15). Biofilm inhibition over 30% was seen for HMOs from three donors (0, 14, and 17), but these values were associated with large fluctuations in SEM such that none were statistically significant. This is likely due to the large fluctuations in biofilm measurements (OD_{560}), which can be attributed to variability in biofilm attachment to the well plate. In THB supplemented with 1% glucose, HMOs from donors 14 and 31 were able to significantly decrease the amount of biofilm produced relative to bacterial growth by 58 and 54%, respectively (Table 3.3, Figure A2.16).

HMOs from donors 0, 5, 14, 19, 24, 29, and 31 significantly decreased the biofilm formation of GB2 in both THB and THB supplemented with 1% glucose from 23-93% (Table 3.3, Figures A2.17 and A2.18). Of note, reduction in biofilm/biomass ratio of 83 and 93% was seen for HMOs from donor 14 in THB and THB supplemented with 1% glucose, respectively. Furthermore, in THB supplemented with 1% glucose, HMOs from donors 8, 17, and 34 also exhibited anti-biofilm activity reducing biofilm/biomass values by 49, 19, and 13%, respectively.

Conclusions to Expanded Study

GBS strain CNCTC 10/84 was most susceptible to the antimicrobial activity of HMOs, showing significant reduction in growth for eight samples compared to only three samples exhibiting antimicrobial activity in GB590 and GB2. GBS strains are classified by their CPS and surface associated proteins. One particular subset of surface associated proteins, surface-anchored alpha-like proteins (Alps), are found in nearly all GBS strains (>98%).[32] Interestingly, CNCTC 10/84 is one of few GBS strains that are considered non-Alp strains because they do not possess

any Alp encoding genes.

Table 3. 3 Anti-biofilm Activity of HMOs against Three Strains of Group B *Streptococcus*[a,b]

		Biofilm/Biomass Change from Control (%)					
		CNCTC 10/84		**GB590**		**GB2**	
Donor	**Lewis Blood Group**	**THB**	**THB +1%Glc**	**THB**	**THB +1%Glc**	**THB**	**THB +1%Glc**
0	a-b+	**-67 ± 11** [b]	-32 ± 13	-40 ± 28	-26 ± 6	**-28 ± 1** [b]	**-45 ± 3**
5	a-b+	**-80 ± 7** [a]	-1 ± 8	-17 ± 35	-19 ± 8	**-51 ± 6** [b]	**-45 ± 3**
7	a-b+	-33 ± 13	**-36 ± 11**	-23 ± 22	-24 ± 5	+10 ± 37	-6 ± 4
8	a-b+	+346 ± 229	-5 ± 17	+178 ± 115	-21 ± 7	+273 ± 71	**-49 ± 5**
14	a-b+	**-63 ± 13** [b]	**-38 ± 11**	-46 ± 18	**-58 ± 5**	**-93 ± 4** [b]	**-83 ± 1**
19	a-b+	**-71 ± 7** [b]	-23 ± 16	-10 ± 54	-28 ± 5	**-40 ± 10** [b]	**-51 ± 2**
24	a-b+	**-70 ± 8** [b]	**-81 ± 3**	0 ± 46	-42 ± 10	**-70 ± 9** [b]	**-33 ± 4**
32	a-b+	**-79 ± 6** [b]	-21 ± 12	-13 ± 44	-20 ± 6	+31 ± 25	-6 ± 3
34	a-b+	-37 ± 16	-20 ± 8	+11 ± 32	+5 ± 7	+8 ± 24	**-13 ± 3**
37	a-b+	**-53 ± 11** [b]	+34 ± 14	+22 ± 35	-5 ± 3	+39 ± 28	-10 ± 3
17	a+b-	**-65 ± 7** [b]	-20 ± 8	-35 ± 17	-11 ± 3	+11 ± 24	**-19 ± 3**
18	a+b-	-38 ± 18	**-40 ± 12**	-18 ± 40	-18 ± 3	**-53 ± 21** [b]	+7 ± 5
29	a+b-	**-60 ± 8** [b]	-27 ± 12	-3 ± 52	+80 ± 31	**-37 ± 12** [b]	**-23 ± 5**
31	a+b-	-33 ± 15	**-43 ± 9**	-23 ± 25	**-54 ± 5**	**-43 ± 10** [b]	**-69 ± 2**

[a]Significant growth inhibition ($p \leq 0.05$, one-way ANOVA) compared to control is boldfaced. [b]Statistically significant activity when results from Donor 8 were omitted; Donor 8 was determined to be an outlier by both ROUT and Grubbs tests.

Alp proteins are useful for virulence because they function as adhesins to adhere to cell surfaces.[33, 34] Perhaps the lack of Alp proteins in CNCTC 10/84 and its need to employ other modes

of virulence for its infectivity make this strain more susceptible to the antimicrobial activity of HMOs. It is of note that CNCTC 10/84 is known as a hypervirulent strain, and the increased antimicrobial effects of HMOs against CNCTC 10/84 are encouraging since this strain belongs to the third most common serotype associated with infant infections caused by GBS.

All HMO samples were found to significantly reduce biofilm production in at least one GBS strain. In several cases, biofilm inhibition was over 70%, demonstrating the ability of HMOs to suppress this form of GB virulence.

CNCTC 10/84 and GB2 appeared to be particularly susceptible to changes in biofilm as over ten HMO samples significantly reduced biofilm formation in both strains, compared to only two HMO samples for GB590. However, GB590 also seemed particularly susceptible, but due to large fluctuations in biofilm measurements attributable to weak or inconsistent attachment of biofilms to the well-plate surface, few decreases in biofilm formation of GB590 were deemed significant.

We have demonstrated that HMOs from a broad range of donors can inhibit both the growth and biofilm formation of GBS. Our initial hypothesis when we began this study was that the Lewis blood groups would correlate with antimicrobial or anti-biofilm activity. However, assaying three GBS strains against 14 donor samples has not revealed a relationship between biological activity and Lewis blood group. Instead, both the data from this expanded study and the initial study suggest that HMOs from secretors and nonsecretors belonging to all Lewis blood groups generally demonstrate comparable levels of biological activity. While Lewis blood groups are associated with linkage-specific fucosyl transferases, the concentration of HMOs can change over the course of lactation.[23, 35] A systemic review by Stahl and coworkers that analyzed the concentrations of

oligosaccharides in previously published studies noted that secretor status, and thus Lewis blood groups, could also influence HMO concentration.[28] Their analysis concluded that nonsecretor mothers produced higher concentrations of HMOs than secretor mothers.[28, 36-38] Specifically, the milks from nonsecretor mothers had higher concentrations of nonfucosylated and α-1,3- and α-1,4-fucosylated HMOs, whereas secretor mothers had higher concentration of α-1,2-fucosylated HMOs. The variability over the course of lactation and inherent increases in certain HMOs could account for the broad antimicrobial and anti-biofilm activity against GBS of HMO samples from donors of all Lewis blood groups used in our study.

As HMO concentration and expression can change over the course of lactation, it is possible that the extreme antimicrobial activity of the HMOs from donor 8, particularly when compared to that of the other donors tested, is due to when in the lactation period the sample was collected. HMO concentration is highest in colostrum and several reports have shown higher concentrations of α-1,2 fucosylated HMOs, such as 2′ -FL, in this early milk.[24, 35, 39] It is possible that milk from donor 8 was collected at an earlier lactation stage than the other samples and thus has larger quantities of certain HMOs that are particularly protective against GBS. Due to de-identification, it is difficult to confidently assign reasons for the marked effects of this sample.

Summary and Conclusions

Overall, we have shown that HMOs demonstrate the ability to modulate both the bacterial growth and biofilm production of several GBS strains. HMOs exhibited up to 89% growth inhibition and up to 93% inhibition of biofilm production (Table 3.4). This work also resulted in the first example of HMOs serving as anti-biofilm agents.

Table 3. 4 Summary of Extent of Growth and Biofilm Inhibition against Three GBS strains

S. agalactiae Strain (Serotype)	CNCTC 10/84 (V)	GB590 (III)	GB2 (Ia)
Growth Inhibition	Up to 89%	Up to 75%	Up to 89%
Biofilm Inhibiton	Up to 81%	Up to 58%	Up to 93%

This data adds to previous studies supporting the importance and potential inhibitory effect of HMOs in defending against GBS colonization. HMOs serve as a protective measure available from the host to decrease risk of GBS transmission. Previously, human milk-based biologics have demonstrated modulatory effects on bacterial biofilm formation. For example, in enteric pathogens such as *E. coli* and *Bacteroides*, sIgA and Mucin increased biofilm formation.[40] Both sIgA and Mucins bind and agglutinate these particular bacterial species, and agglutination is postulated as a prerequisite for biofilm formation. In another study with *S. mutans*, lactoferrin and IgA were shown to inhibit biofilm formation while lactose and casein enhance biofilm formation.[41] The variability of the effects of human milk components on different bacterial species could be due to the individual metabolic capabilities of bacteria. Genome analysis of several *streptococci* species (*S. pyogenes, S. pneumoniae, and S. agalactiae*) has shown that while *S. pyogenes* and *S. pneumoniae* have systems to metabolize fucose, lactose, mannitol, and raffinose, some strains of *S. agalactiae* lack these metabolic systems.[42] We speculate the anti-biofilm activity of HMOs could be related to metabolism and the presence of complex, long-chain human milk oligosaccharides, which the bacteria cannot use as direct nutrients but may recognize these oligosaccharides as surrogates for "wild-type" bacterial polysaccharides as they are structurally similar.[20, 43-45] This mechanism could coerce the bacteria into decreasing production of other biofilm forming components. From the standpoint of bacterial pathogenesis, the ability to construct

and maintain a structured, multicellular bacterial community depends on the production of extracellular matrix components. Microbes produce complex biofilm matrices consisting of proteins, extracellular DNA, and polysaccharides. Polysaccharide overproduction can alter the morphology of a colony.[20, 43, 46]

Furthermore, our work indicates HMOs can inhibit bacterial growth. These effects appeared to be largely dependent on the nutritional composition of the growth medium. While biofilm formation is less affected by the media content, growth inhibition is decreased when THB is supplemented with glucose. Carbohydrate catabolism has been implicated as a critical step in the pathogenesis of *streptococcal* disease as a number of mechanisms (e.g., initiation of virulence factors) are closely associated with the ability of streptococci to use glucose.[47] GBS is known to utilize excess glucose to increase replication and lower the pH of growth media.[18, 48] We hypothesize that, in the case of GBS, glucose supplementation increases bacterial proliferation thereby assisting the bacteria to avert effects of exposure to HMOs. As seen in the growth curves of GBS grown in THB supplemented with glucose and HMOs from donor 43, there is an intense period of growth repression (Figure 3.3, t = 0-22 h). This potentially reflects a period where GBS is slowly proliferating and working to achieve a new balance of metabolic reactions due to the additional carbohydrate sources (HMOs). The approach to achieving a new balance may involve both changes in concentrations of metabolic intermediates and in the relative amounts of various enzymes during adaptation to a new carbon source.[49] Interestingly, genetic analyses in related *streptococcal* species, such as *S. mutans*, reveal that accumulation of galactose metabolism intermediates can inhibit bacterial growth.[50] Thus, it remains possible that exposure of GBS to HMOs may serve to alter carbohydrate metabolism leading to accumulation of toxic intermediates which ultimately repress bacterial growth or biofilm formation. This hypothesis could be tested by

examining cell-free media extracts for the evidence of carbohydrate utilization. Specifically, measuring the pH of the media can indicate the presence of organic acids that are a by-product of carbohydrate fermentation. Additionally, metabolomics and genetic analysis through RNA sequencing could reveal what changes are associated with growth of GBS in the presence of HMOs.

A recent study conducted by Bode and coworkers showed the narrow-spectrum antimicrobial activity of HMOs to inhibit GBS growth.[17] A library of GBS mutants was generated to probe the mechanism of this action. Only one out of 1300 mutants was found that overcame the antimicrobial activity of HMOs. This mutant lacked gbs0738 gene, which was postulated to encode for a glycosyltransferase that catalyzes incorporation of HMOs into the cell wall, causing the antimicrobial activity. While the exact use of this gene was not elucidated in their study, further literature review revealed that gbs0738 gene has an identical sequence to gbs0408 gene.[51, 52] A report by Glaser and coworkers identified gbs0408 to be part of TnGBS1 and to encode for a protein responsible for initiating replication of plasmids or other mobile genetic elements (MGE).[53] TnGBS is an integrative and conjugative element (ICE), which is part of a larger category of mobile genetic elements (MGE). While ICE are not a permanent part of the host genone, they may represent a significant portion.[53, 54] For instance, in the bacterium *Orientia tsutsugamushi*, ICE account for over 30% of the genome and in one *E. coli* strain, 15% of the genome is composed of ICE.[53, 55, 56] ICE are postulated to carry genes that provide advantages for host colonization such as specific metabolite functions, drug resistance, or virulence factors.[53, 54] Replication of TnGBS is correlated with transfer efficiency between cells, and the ability to regulate the activity of gbs0408-encoded protein could affect the ability of this ICE to be transferred to progeny bacteria, causing them to lose some of the virulence associated with the TnGBS gene cluster.[53, 57, 58] More

specifically for the context of the anti-biofilm activity of HMOs, the evidence that gbs0408 gene plays a role in HMO activity could be related to the transfer of this genetic element to progeny bacteria. Research programs led by Gilot and Jenkinson have suggested that TnGBS regulates gene expression, biofilm formation, host colonization, and immunomodulation, leading us to hypothesize that this MGE is influenced by the presence of HMOs and is unable to replicate, rendering the bacteria unable to proliferate or produce biofilm as efficiently.[57, 59, 60]

These studies examined the effect of whole carbohydrate extracts on GBS. As we found no significant difference between Lewis blood group classification of the mother and HMOs on the growth or biofilm formation of GBS, we postulate that future studies toward the effects of individual HMOs on GBS may uncover unique pharmacophores responsible for HMO antimicrobial and anti-biofilm properties.

Methods

Bacterial Strains and Culture Conditions

S. agalactiae strain CNCTC 10/84 (ATCC) was cultured on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37 °C in ambient air overnight. Bacteria were subcultured from blood agar plates into Todd-Hewitt broth (THB) and incubated under shaking conditions at 37 °C in ambient air overnight. The following day, bacterial density was measured spectrophotometrically using optical density measurements at 600 nm (OD_{600}), and bacterial numbers were determined using the predetermined coefficient of 1 $OD_{600} = 10^9$ CFU/mL.

Bacterial Growth and Viability Analyses

S. agalactiae strain CNCTC 10/84 cells were cultured overnight in THB and then subcultured by inoculating 106 cells per 5 mL of THB or THB supplemented with 1% glucose.

Cultures were grown under shaking conditions in THB alone or supplemented with 5 mg/mL HMOs isolated from the various human milk samples (Donors 16, 20, 38, 42, and 43) or in THB supplemented with 1% glucose or THB supplemented with 1% glucose and 5 mg/mL HMOs isolated from the various human milk samples (Donors 16, 20, 38, 42, and 43) at 37 °C in ambient air. Bacterial growth was evaluated by spectrophotometric reading of OD_{600}, and bacterial viability was evaluated by serial dilution and plating onto blood agar plates and quantifying viable colony forming units per mL of culture (CFU/mL).

Bacterial Biofilm Assays

S. agalactiae strain CNCTC 10/84 was grown overnight as described above prior to subculturing 10^6 bacterial cells into 200 μL of THB supplemented with 1% glucose (to promote biofilm formation) in 96-well tissue culture plates (Corning, Inc.). Bacterial cells were added to wells containing media alone or wells supplemented with 5 mg/mL HMOs isolated from the various human milk samples (Donors 16, 20, 38, 42, and 43). Cultures were incubated under static conditions at 37 ° C in ambient air for 24 h. Optical density (OD_{600}) was measured for each sample as a measure of bacterial growth. The medium was aspirated, and each well was washed once with phosphate-buffered saline (PBS, pH 7.4) to remove nonadherent cells. Wells were then stained with a 10% crystal violet solution for 15 min. After staining, the wells were again washed with PBS and then allowed to dry at room temperature for 30 min. After drying, crystal violet staining was solubilized with a 4:1 ethanol/acetone solution. The absorbance (OD_{560}) was measured for each sample as a measure of biofilm formation. The data shown represents 5 independent experiments, each with 3 technical replicates.

Field-Emission Gun Scanning Electron Microscopy

Bacterial cells were analyzed by scanning electron microscopy. Bacteria were cultured in THB supplemented with 1% glucose in wells containing 12 mm glass coverslips coated with poly-L lysine (Corning, Bedford MA) at 37 ° C for 24 h. At 24 h, supernatants were removed, and samples were fixed with 2.0% paraformaldehyde and 2.5% gluteraldehyde in 0.05 M sodium cacodylate buffer for 24 h. Secondary fixation with 0.1% osmium tetroxide was performed for 5 min prior to sequential dehydration with increasing concentrations of ethanol. After ethanol dehydration, samples were dried at the critical point using a critical point dryer machine (Tousimis), mounted onto aluminum sample stubs, and sputter-coated with 80/20 gold−palladium. Afterward, samples were painted with a thin strip of colloidal silver (Electron Microscopy Sciences) at the edge to facilitate charge dissipation. Samples were imaged with an FEI Quanta 250 field-emission gun scanning electron microscope. Images shown are representative of three separate experiments.

Confocal Laser Scanning Microscopy Analyses

Bacterial cells were cultured as above in wells containing THB supplemented with 1% glucose and containing glass coverslips coated with poly-L-lysine. Cultures were grown under static conditions for 24 h at 37 ° C. At 24 h, coverslips were washed with PBS prior to staining with a LIVE/DEAD BacLight bacterial viability kit, which includes both SYTO 9 (green) and propidium iodide (red) (Life Technologies) to visualize bacterial cells and calcofluor white (blue) (Sigma-Aldrich) to visualize the carbohydrate capsule/matrix within the biofilm. Coverslips were stained for 15 min followed by 2 washes with PBS. Both SYTO 9 and propidium iodide stain nucleic acids, but propidium iodide is only able to penetrate damaged cell membranes and competes with SYTO 9 to stain within dead bacterial cells. When used concurrently, stained

bacteria with intact cell membranes will fluoresce green and those with damaged cells will fluoresce red. Calcofluor white binds to β-1,3 and β-1,4 polysaccharides such as chitin and cellulose and has been shown to stain the extrapolymeric substances in biofilms of Streptococcus species and other bacteria. Coverslips were then mounted on glass microscope slides using Aqua Poly/Mount (Polysciences, Inc.). Samples were imaged with a Zeiss LSM 710 Meta Inverted confocal laser-scanning microscope with Zen 2011 software.

References

1. Jantscher-Krenn, E.; Lauwaet, T.; Bliss, L. A.; Reed, S. L.; Gillin, F. D.; Bode, L., Human milk oligosaccharides reduce Entamoeba histolytica attachment and cytotoxicity in vitro. *Br J Nutr* **2012,** *108*, 1839-46.

2. Idanpaan-Heikkila, I.; Simon, P. M.; Zopf, D.; Vullo, T.; Cahill, P.; Sokol, K.; Tuomanen, E., Oligosaccharides interfere with the establishment and progression of experimental pneumococcal pneumonia. *J Infect Dis* **1997,** *176*, 704-12.

3. Martin-Sosa, S.; Martin, M. J.; Hueso, P., The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic Escherichia coli human strains. *J Nutr* **2002,** *132*, 3067-72.

4. Lin, A. E.; Autran, C. A.; Espanola, S. D.; Bode, L.; Nizet, V., Human milk oligosaccharides protect bladder epithelial cells against uropathogenic Escherichia coli invasion and cytotoxicity. *J Infect Dis* **2014,** *209*, 389-98.

5. Crane, J. K.; Azar, S. S.; Stam, A.; Newburg, D. S., Oligosaccharides from human milk block binding and activity of the Escherichia coli heat-stable enterotoxin (STa) in T84 intestinal cells. *J Nutr* **1994,** *124*, 2358-64.

6. Cravioto, A.; Tello, A.; Villafan, H.; Ruiz, J.; del Vedovo, S.; Neeser, J. R., Inhibition of localized adhesion of enteropathogenic Escherichia coli to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. *J Infect Dis* **1991,** *163*, 1247-55.

7. Ruiz-Palacios, G. M.; Cervantes, L. E.; Ramos, P.; Chavez-Munguia, B.; Newburg, D. S., Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem* **2003,** *278*, 14112-20.

8. Newburg, D. S.; Pickering, L. K.; McCluer, R. H.; Cleary, T. G., Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of Escherichia coli. *J Infect Dis* **1990,** *162*, 1075-80.

9. Morrow, A. L.; Ruiz-Palacios, G. M.; Jiang, X.; Newburg, D. S., Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *J Nutr* **2005,** *135*, 1304-7.

10. Marionneau, S.; Ruvoen, N.; Le Moullac-Vaidye, B.; Clement, M.; Cailleau-Thomas, A.; Ruiz-Palacois, G.; Huang, P.; Jiang, X.; Le Pendu, J., Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. *Gastroenterology* **2002,** *122*, 1967-77.

11. Lindesmith, L.; Moe, C.; Marionneau, S.; Ruvoen, N.; Jiang, X.; Lindblad, L.; Stewart, P.; LePendu, J.; Baric, R., Human susceptibility and resistance to Norwalk virus infection. *Nat Med* **2003,** *9*, 548-53.

12. Marionneau, S.; Airaud, F.; Bovin, N. V.; Le Pendu, J.; Ruvoen-Clouet, N., Influence of the combined ABO, FUT2, and FUT3 polymorphism on susceptibility to Norwalk virus attachment. *J Infect Dis* **2005,** *192*, 1071-7.

13. Newburg, D. S.; Ruiz-Palacios, G. M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero Mde, L.; Morrow, A. L., Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology* **2004,** *14*, 253-63.

14. Newburg, D. S.; Ruiz-Palacios, G. M.; Morrow, A. L., Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr* **2005,** *25*, 37-58.

15. Bode, L., The functional biology of human milk oligosaccharides. *Early Hum Dev* **2015,** *91*, 619-622.

16. Andreas, N. J.; Al-Khalidi, A.; Jaiteh, M.; Clarke, E.; Hyde, M. J.; Modi, N.; Holmes, E.; Kampmann, B.; Mehring Le Doare, K., Role of human milk oligosaccharides in Group B Streptococcus colonisation. *Clin Transl Immunology* **2016,** *5*, e99.

17. Lin, A. E.; Autran, C. A.; Szyszka, A.; Escajadillo, T.; Huang, M.; Godula, K.; Prudden, A. R.; Boons, G. J.; Lewis, A. L.; Doran, K. S.; Nizet, V.; Bode, L., Human milk oligosaccharides inhibit growth of group B Streptococcus. *J Biol Chem* **2017**.

18. Rosini, R.; Margarit, I., Biofilm formation by Streptococcus agalactiae: influence of environmental conditions and implicated virulence factors. *Front Cell Infect Microbiol* **2015,** *5*, 6.

19. Chuzeville, S.; Dramsi, S.; Madec, J. Y.; Haenni, M.; Payot, S., Antigen I/II encoded by integrative and conjugative elements of Streptococcus agalactiae and role in biofilm formation. *Microb Pathog* **2015,** *88*, 1-9.

20. Xia, F. D.; Mallet, A.; Caliot, E.; Gao, C.; Trieu-Cuot, P.; Dramsi, S., Capsular polysaccharide of Group B Streptococcus mediates biofilm formation in the presence of human plasma. *Microbes Infect* **2015,** *17*, 71-6.

21. Pritchard, D. G.; Gray, B. M.; Egan, M. L., Murine monoclonal antibodies to type Ib polysaccharide of group B *streptococci* bind to human milk oligosaccharides. *Infect Immun* **1992,** *60*, 1598-602.

22. Pritchard, D. G.; Egan, M. L.; Gray, B. M.; Dillon, H. C., Jr., Immunochemical characterization of the polysaccharide antigens of group B streptococci. *Rev Infect Dis* **1988,** *10 Suppl 2*, S367-71.

23. Ballard, O.; Morrow, A. L., Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am* **2013,** *60*, 49-74.

24. Asakuma, S.; Urashima, T.; Akahori, M.; Obayashi, H.; Nakamura, T.; Kimura, K.; Watanabe, Y.; Arai, I.; Sanai, Y., Variation of major neutral oligosaccharides levels in human colostrum. *Eur J Clin Nutr* **2008,** *62*, 488-94.

25. Koch, A. L., Turbidity measurements of bacterial cultures in some available commercial instruments. *Anal Biochem* **1970,** *38*, 252-9.

26. Welch, K.; Cai, Y.; Strømme, M., A method for quantitative determination of biofilm viability. *J Funct Biomater* **2012,** *3*, 418-31.

27. Morrow, A. L.; Ruiz-Palacios, G. M.; Altaye, M.; Jiang, X.; Guerrero, M. L.; Meinzen-Derr, J. K.; Farkas, T.; Chaturvedi, P.; Pickering, L. K.; Newburg, D. S., Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J Pediatr* **2004,** *145*, 297-303.

28. Thurl, S.; Munzert, M.; Boehm, G.; Matthews, C.; Stahl, B., Systematic review of the concentrations of oligosaccharides in human milk. *Nutr Rev* **2017,** *75*, 920-933.

29. Blank, D.; Gebhardt, S.; Maass, K.; Lochnit, G.; Dotz, V.; Blank, J.; Geyer, R.; Kunz, C., High-throughput mass finger printing and Lewis blood group assignment of human milk oligosaccharides. *Anal Bioanal Chem* **2011,** *401*, 2495-510.

30. Madrid, L.; Seale, A. C.; Kohli-Lynch, M.; Edmond, K. M.; Lawn, J. E.; Heath, P. T.; Madhi, S. A.; Baker, C. J.; Bartlett, L.; Cutland, C.; Gravett, M. G.; Ip, M.; Le Doare, K.; Rubens, C. E.; Saha, S. K.; Sobanjo-Ter Meulen, A.; Vekemans, J.; Schrag, S.; Group, I. G. D. I., Infant Group B *Streptococcal* Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **2017,** *65*, S160-S172.

31. Di Palo, B.; Rippa, V.; Santi, I.; Brettoni, C.; Muzzi, A.; Metruccio, M. M.; Grifantini, R.; Telford, J. L.; Paccani, S. R.; Soriani, M., Adaptive response of Group B streptococcus to high glucose conditions: new insights on the CovRS regulation network. *PLoS One* **2013,** *8*, e61294.

32. Gabrielsen, C.; Mæland, J. A.; Lyng, R. V.; Radtke, A.; Afset, J. E., Molecular characteristics of Streptococcus agalactiae strains deficient in alpha-like protein encoding genes. *J Med Microbiol* **2017,** *66*, 26-33.

33. Baron, M. J.; Bolduc, G. R.; Goldberg, M. B.; Aupérin, T. C.; Madoff, L. C., Alpha C protein of group B Streptococcus binds host cell surface glycosaminoglycan and enters cells by an actin-dependent mechanism. *J Biol Chem* **2004,** *279*, 24714-23.

34. Baron, M. J.; Filman, D. J.; Prophete, G. A.; Hogle, J. M.; Madoff, L. C., Identification of a glycosaminoglycan binding region of the alpha C protein that mediates entry of group B Streptococci into host cells. *J Biol Chem* **2007,** *282*, 10526-36.

35. Kunz, C.; Meyer, C.; Collado, M. C.; Geiger, L.; García-Mantrana, I.; Bertua-Ríos, B.; Martínez-Costa, C.; Borsch, C.; Rudloff, S., Influence of Gestational Age, Secretor, and Lewis Blood Group Status on the Oligosaccharide Content of Human Milk. *J Pediatr Gastroenterol Nutr* **2017,** *64*, 789-798.

36. Thurl, S.; Munzert, M.; Henker, J.; Boehm, G.; Müller-Werner, B.; Jelinek, J.; Stahl, B., Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr* **2010,** *104*, 1261-71.

37. Smilowitz, J. T.; O'Sullivan, A.; Barile, D.; German, J. B.; Lönnerdal, B.; Slupsky, C. M., The human milk metabolome reveals diverse oligosaccharide profiles. *J Nutr* **2013,** *143*, 1709-18.

38. Gabrielli, O.; Zampini, L.; Galeazzi, T.; Padella, L.; Santoro, L.; Peila, C.; Giuliani, F.; Bertino, E.; Fabris, C.; Coppa, G. V., Preterm milk oligosaccharides during the first month of lactation. *Pediatrics* **2011,** *128*, e1520-31.

39. Urashima, T.; Asakuma, S.; Leo, F.; Fukuda, K.; Messer, M.; Oftedal, O. T., The Predominance of Type I Oligosaccharides Is a Feature Specific to Human Breast Milk. *Adv Nutr* **2012,** *3*, 473s-482s.

40. Bollinger, R. R.; Everett, M. L.; Palestrant, D.; Love, S. D.; Lin, S. S.; Parker, W., Human secretory immunoglobulin A may contribute to biofilm formation in the gut. *Immunology* **2003,** *109*, 580-7.

41. Allison, L. M.; Walker, L. A.; Sanders, B. J.; Yang, Z.; Eckert, G.; Gregory, R. L., Effect of Human Milk and its Components on *Streptococcus* Mutans Biofilm Formation. *J Clin Pediatr Dent* **2015,** *39*, 255-61.

42. Tettelin, H.; Masignani, V.; Cieslewicz, M. J.; Eisen, J. A.; Peterson, S.; Wessels, M. R.; Paulsen, I. T.; Nelson, K. E.; Margarit, I.; Read, T. D.; Madoff, L. C.; Wolf, A. M.; Beanan, M. J.; Brinkac, L. M.; Daugherty, S. C.; DeBoy, R. T.; Durkin, A. S.; Kolonay, J. F.; Madupu, R.; Lewis, M. R.; Radune, D.; Fedorova, N. B.; Scanlan, D.; Khouri, H.; Mulligan, S.; Carty, H. A.; Cline, R. T.; Van Aken, S. E.; Gill, J.; Scarselli, M.; Mora, M.; Iacobini, E. T.; Brettoni, C.; Galli, G.; Mariani, M.; Vegni, F.; Maione, D.; Rinaudo, D.; Rappuoli, R.; Telford, J. L.; Kasper, D. L.; Grandi, G.; Fraser, C. M., Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V Streptococcus agalactiae. *Proc Natl Acad Sci U S A* **2002,** *99*, 12391-6.

43. Arciola, C. R.; Campoccia, D.; Ravaioli, S.; Montanaro, L., Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. *Front Cell Infect Microbiol* **2015,** *5*, 7.

44. Murphy, K.; Park, A. J.; Hao, Y.; Brewer, D.; Lam, J. S.; Khursigara, C. M., Influence of O polysaccharides on biofilm development and outer membrane vesicle biogenesis in Pseudomonas aeruginosa PAO1. *J Bacteriol* **2014,** *196*, 1306-17.

45. Zdorovenko, E. L.; Shashkov, A. S.; Zhurina, M. V.; Plakunov, V. K.; Knirel, Y. A., Structure of the O-specific polysaccharides from planktonic and biofilm cultures of Pseudomonas chlororaphis 449. *Carbohydr Res* **2015,** *404*, 93-7.

46. Limoli, D. H.; Jones, C. J.; Wozniak, D. J., Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. *Microbiol Spectr* **2015,** *3*.

47. Almengor, A. C.; Kinkel, T. L.; Day, S. J.; McIver, K. S., The catabolite control protein CcpA binds to Pmga and influences expression of the virulence regulator Mga in the Group A streptococcus. *J Bacteriol* **2007,** *189*, 8405-16.

48. D'Urzo, N.; Martinelli, M.; Pezzicoli, A.; De Cesare, V.; Pinto, V.; Margarit, I.; Telford, J. L.; Maione, D.; Group, M. o. t. D. S., Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 Streptococcus agalactiae strains. *Appl Environ Microbiol* **2014,** *80*, 2176-85.

49. Mickelson, M., Glucose-Transport in *Streptococcus Agalactiae* and its Inhibition by Lactoperoxidase-Thiocyanate-Hydrogen Peroxide. *J Bacteriol* **1977,** *132*, 541-548.

50. Zeng, L.; Das, S.; Burne, R. A., Utilization of lactose and galactose by *Streptococcus* mutans: transport, toxicity, and carbon catabolite repression. *J Bacteriol* **2010,** *192*, 2434-44.

51. **hypothetical protein gbs0738 [Streptococcus agalactiae NEM316]**. National Library of Medicine (US), National Center for Biotechnology Information: Bethesda, MD.

52. **hypothetical protein [Streptococcus agalactiae], NCBI Reference Sequence: WP_000323828.1**. 14-MAY-2013 ed. National Library of Medicine (US), National Center for Biotechnology Information: Bethesda, MD.

53. Guérillot, R.; Da Cunha, V.; Sauvage, E.; Bouchier, C.; Glaser, P., Modular evolution of TnGBSs, a new family of integrative and conjugative elements associating insertion sequence transposition, plasmid replication, and conjugation for their spreading. *J Bacteriol* **2013,** *195*, 1979-90.

54. Johnson, C. M.; Grossman, A. D., Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annu Rev Genet* **2015,** *49*, 577-601.

55. Zhou, Y.; Liang, Y.; Lynch, K. H.; Dennis, J. J.; Wishart, D. S., PHAST: a fast phage search tool. *Nucleic Acids Res* **2011,** *39*, W347-52.

56. Nakayama, K.; Yamashita, A.; Kurokawa, K.; Morimoto, T.; Ogawa, M.; Fukuhara, M.; Urakami, H.; Ohnishi, M.; Uchiyama, I.; Ogura, Y.; Ooka, T.; Oshima, K.; Tamura, A.; Hattori, M.; Hayashi, T., The Whole-genome sequencing of the obligate intracellular

bacterium Orientia tsutsugamushi revealed massive gene amplification during reductive genome evolution. *DNA Res* **2008,** *15*, 185-99.

57. Fléchard, M.; Gilot, P., Physiological impact of transposable elements encoding DDE transposases in the environmental adaptation of Streptococcus agalactiae. *Microbiology* **2014,** *160*, 1298-315.

58. Weaver, K. E.; Kwong, S. M.; Firth, N.; Francia, M. V., The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids. *Plasmid* **2009,** *61*, 94-109.

59. Brady, L. J.; Maddocks, S. E.; Larson, M. R.; Forsgren, N.; Persson, K.; Deivanayagam, C. C.; Jenkinson, H. F., The changing faces of Streptococcus antigen I/II polypeptide family adhesins. *Mol Microbiol* **2010,** *77*, 276-86.

60. Maddocks, S. E.; Wright, C. J.; Nobbs, A. H.; Brittan, J. L.; Franklin, L.; Strömberg, N.; Kadioglu, A.; Jepson, M. A.; Jenkinson, H. F., Streptococcus pyogenes antigen I/II-family polypeptide AspA shows differential ligand-binding properties and mediates biofilm formation. *Mol Microbiol* **2011,** *81*, 1034-49.

Appendix A2:

Figures and Supplemental Data Relevant to Chapter III

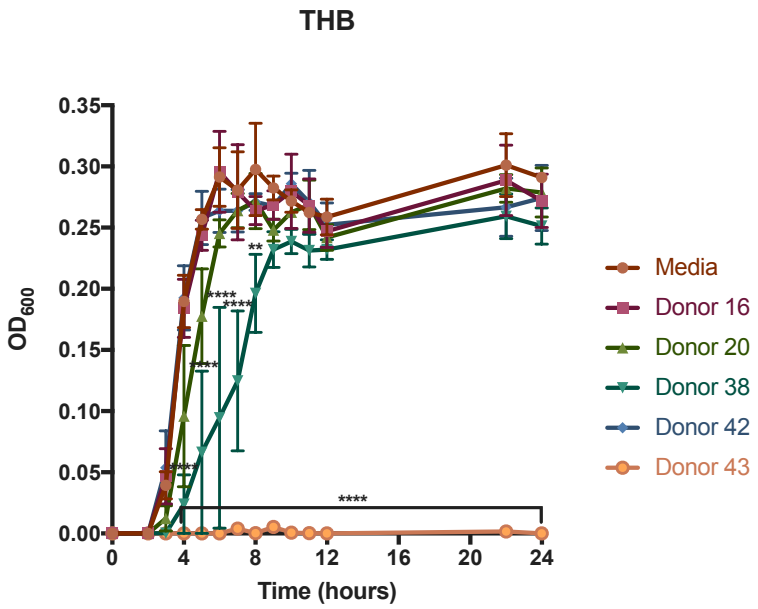


Figure A2. 1 Effect of HMOs isolated from individual milk samples on growth rate/proliferation of GBS CNCTC 10/84 in Todd Hewitt Broth. OD_{600} readings were taken at 0, 2−12, 22, and 24 h. Mean OD_{600} for each HMO sample and time point is indicated by the respective symbols. Data displayed represents the mean OD ± SEM of 3 biological replicates, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by 2-way ANOVA with posthoc Dunnett's multiple comparison test, with all donor samples compared to the GBS growth in media alone.

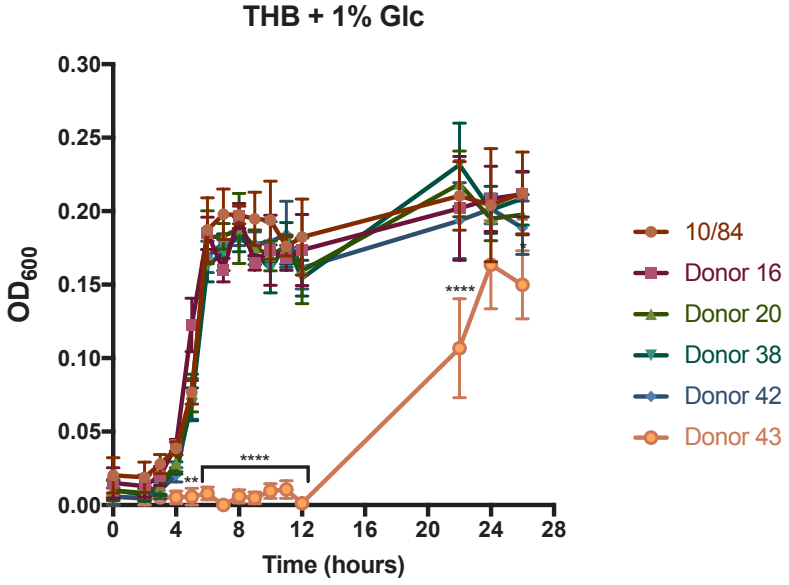


Figure A2. 2 Effect of HMOs isolated from individual milk samples on growth rate/proliferation of GBS CNCTC 10/84 in Todd Hewitt Broth supplemented with 1% glucose. OD_{600} readings were taken at 0, 2−12, 22, 24, and 26 h. Mean OD_{600} for each HMO sample and time point is indicated by the respective symbols. Data displayed represents the mean OD ± SEM of 3 biological replicates, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by 2-way ANOVA with posthoc Dunnett's multiple comparison test, with all donor samples compared to the GBS growth in media alone.

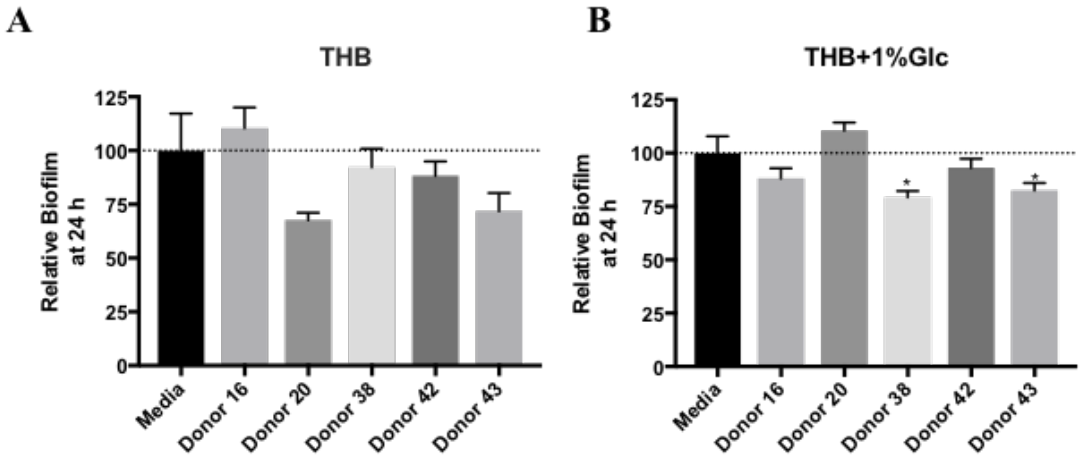


Figure A2. 3 HMOs at biologically relevant breast milk concentrations induce changes in biofilm formation of GBS cultures. The total biofilm measured after 24 h of growth was compared for (A) THB medium alone. Data represented as the mean biofilm/biomass ratio ± SEM of 5 separate experiments, each with 3 technical replicates. There was no statistical significance for GBS grown in THB. (B) THB medium supplemented with 1% glucose. Data are expressed as the mean biofilm/biomass ratio ± SEM of 5 separate experiments, each with 3 technical replicates. * represents $p < 0.05$ by oneway ANOVA, F = 5.935 with posthoc Dunnet's multiple comparison, compared to media alone.

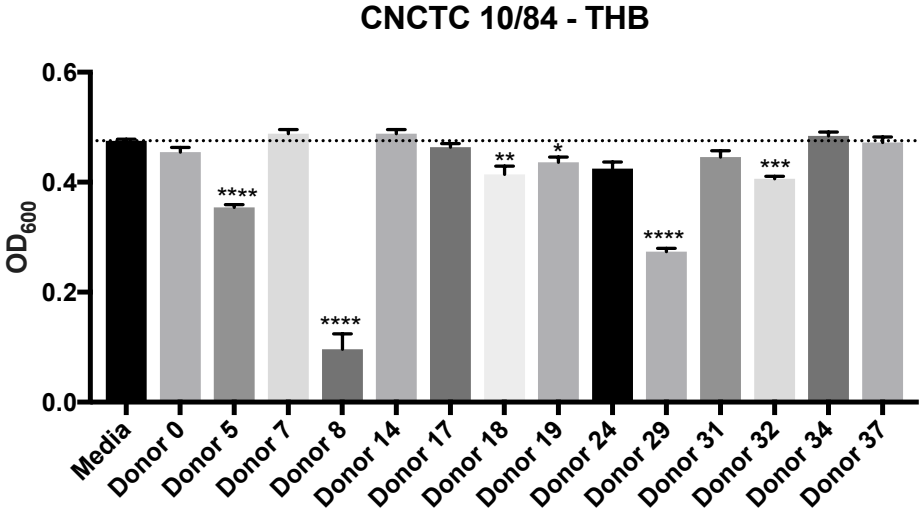


Figure A2.4 Biomass for *S. agalactiae* strain CNCTC 10/84 after 24 h of growth in THB media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data represented as the mean biomass (OD_{600}) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = 88.34 with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

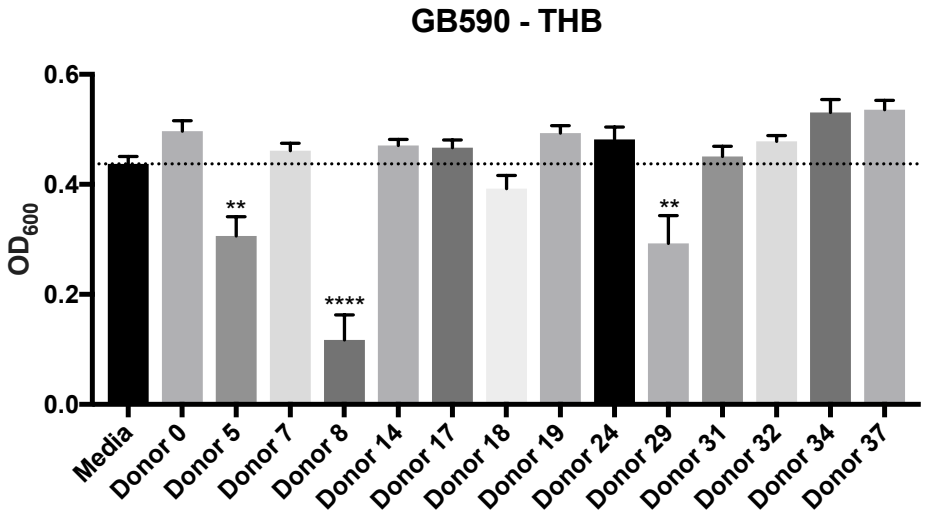


Figure A2.5 Biomass for *S. agalactiae* strain GB590 after 24 h of growth in THB media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data represented as the mean biomass (OD_{600}) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 19.55$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

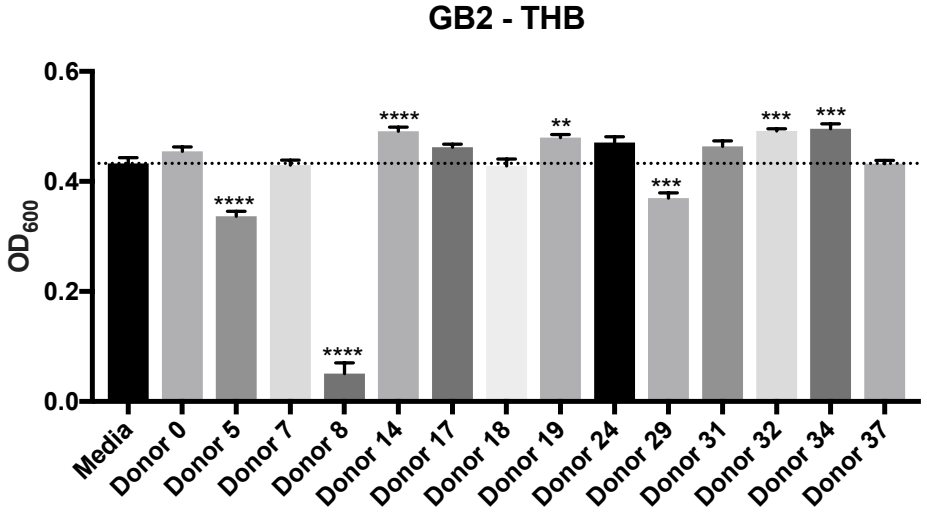


Figure A2.6 Biomass for S. agalactiae strain GB2 after 24 h of growth in THB media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data represented as the mean biomass (OD600) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 132.3$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

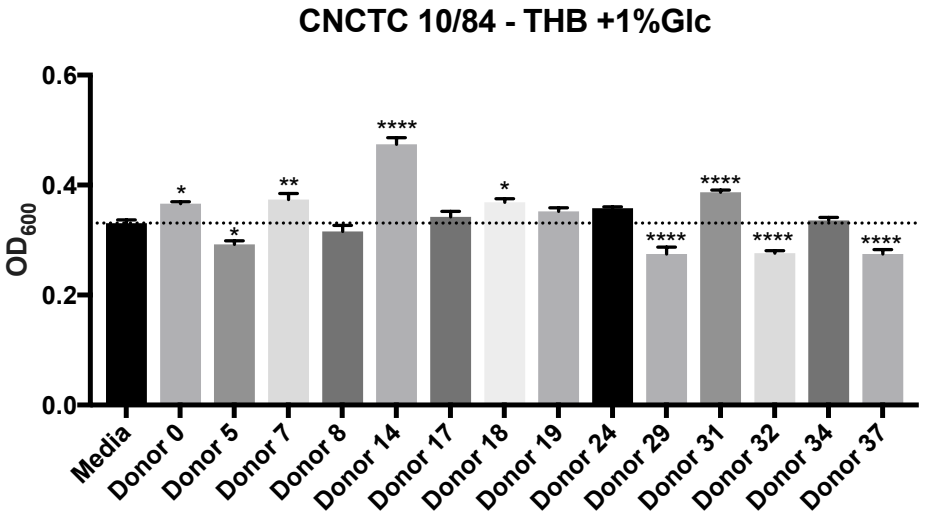


Figure A2.7 Biomass for S. agalactiae strain CNCTC 10/84 after 24 h of growth in THB + 1% glucose media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data represented as the mean biomass (OD600) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = 43.21 with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

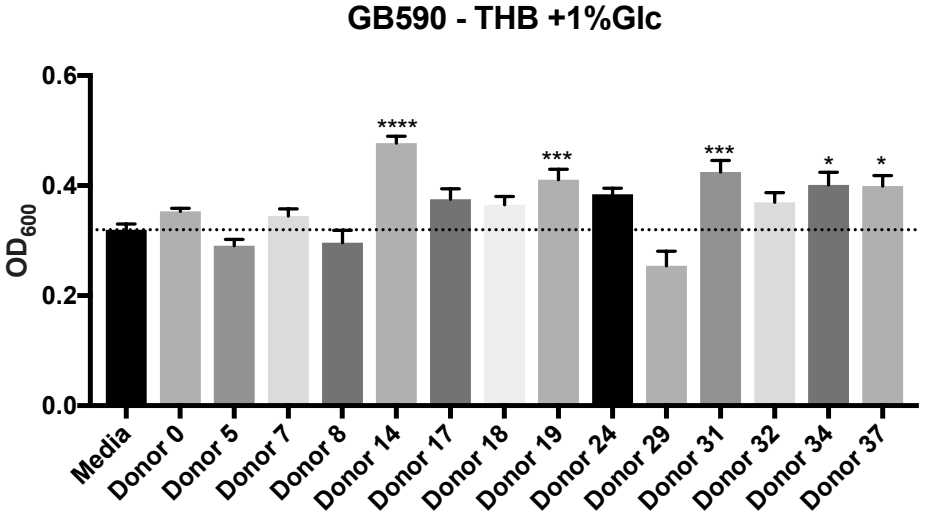


Figure A2.8 Biomass for S. agalactiae strain GB590 after 24 h of growth in THB + 1% glucose media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data represented as the mean biomass (OD600) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = 10.53 with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

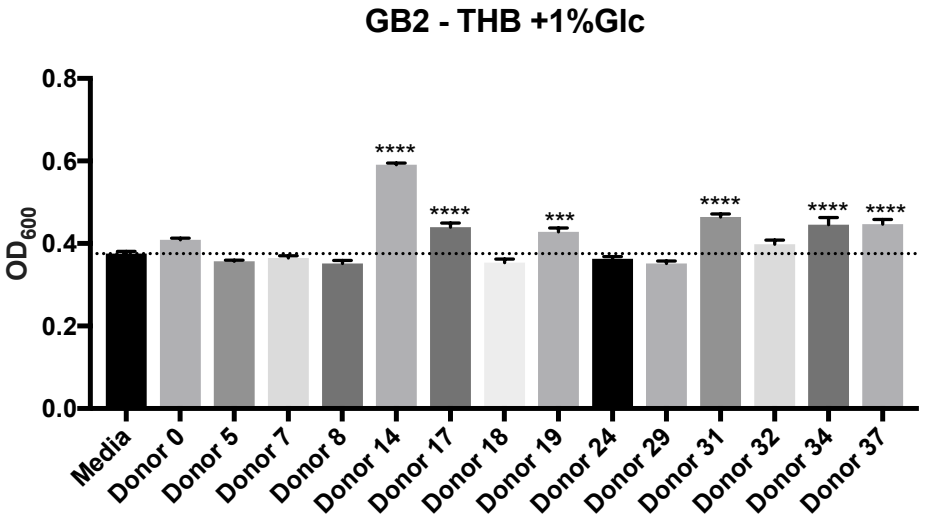


Figure A2.9 Biomass for S. agalactiae strain GB2 after 24 h of growth in THB + 1% glucose media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data represented as the mean biomass (OD600) ± SEM of 3 separate experiments, each with 3 technical replicates. * represents $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 58.52$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

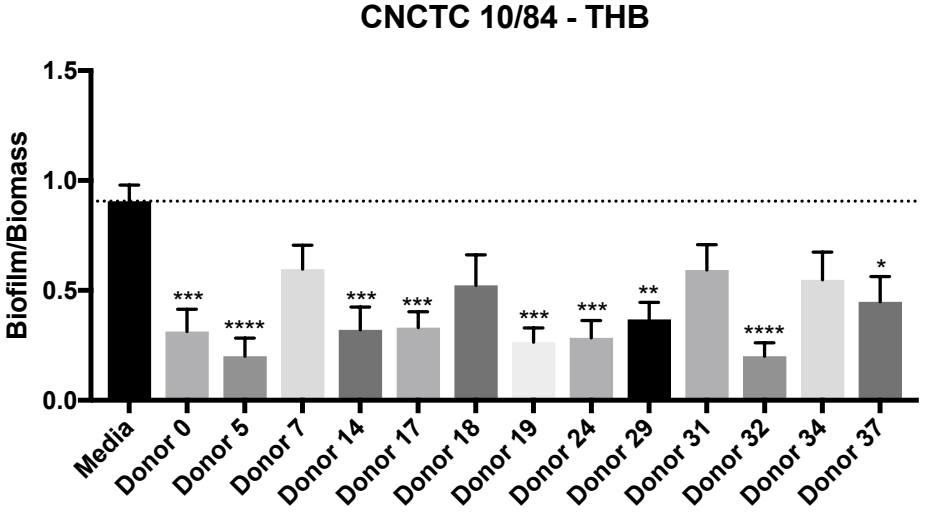


Figure A2.10 Biofilm to biomass ratio for S. agalactiae strain CNCTC 10/84 after 24 h of growth in THB media alone or in the presence of ca. 5 mg/mL HMOs from various donors excluding Donor 8. Data expressed as mean biofilm/biomass ratio measurements (OD560 /OD600) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 4.065$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs. Results from Donor 8 were determined to be outliers using ROUT (Q = 1) and Grubbs (alpha = 0.05) outlier tests.

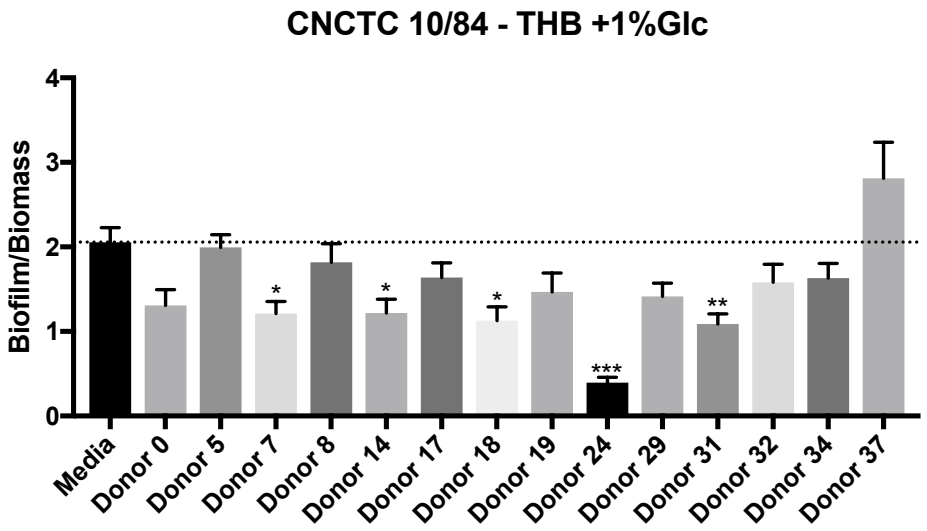


Figure A2.11 Biofilm to biomass ratio of S. agalactiae strain CNCTC 10/84 after 24 h of growth in THB + 1% glucose media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data expressed as mean biofilm/biomass ratio measurements (OD560/OD600) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 7.579$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

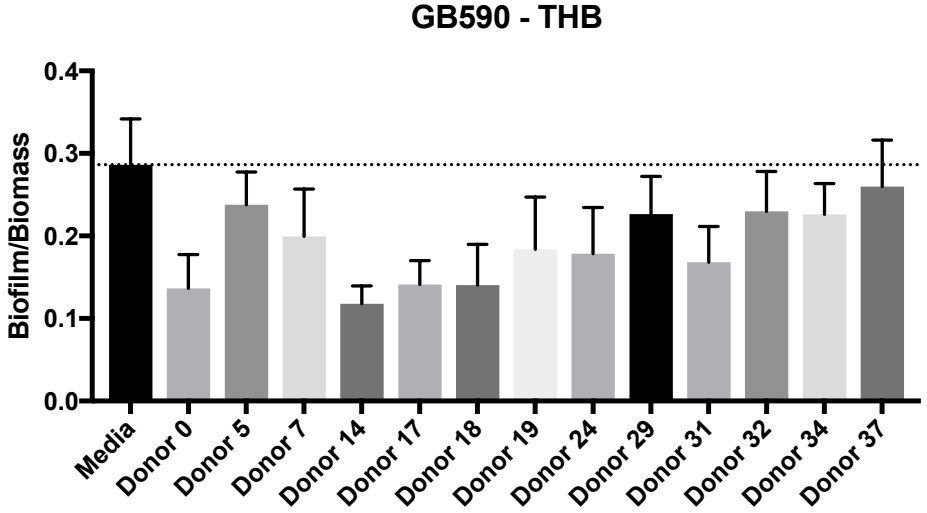


Figure A2.12 Biofilm to biomass ratio for *S. agalactiae* strain GB590 after 24 h of growth in THB media alone or in the presence of ca. 5 mg/mL HMOs from various donors excluding Donor 8. Data expressed as mean biofilm/biomass ratio measurements (OD_{560}/OD_{600}) ± SEM of 3 separate experiments, each with 3 technical replicates. No results were found to be significant by one-way ANOVA, $F = 1.061$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs. Results from Donor 8 were determined to be outliers using ROUT (Q=1) and Grubbs (alpha=0.05) outlier tests.

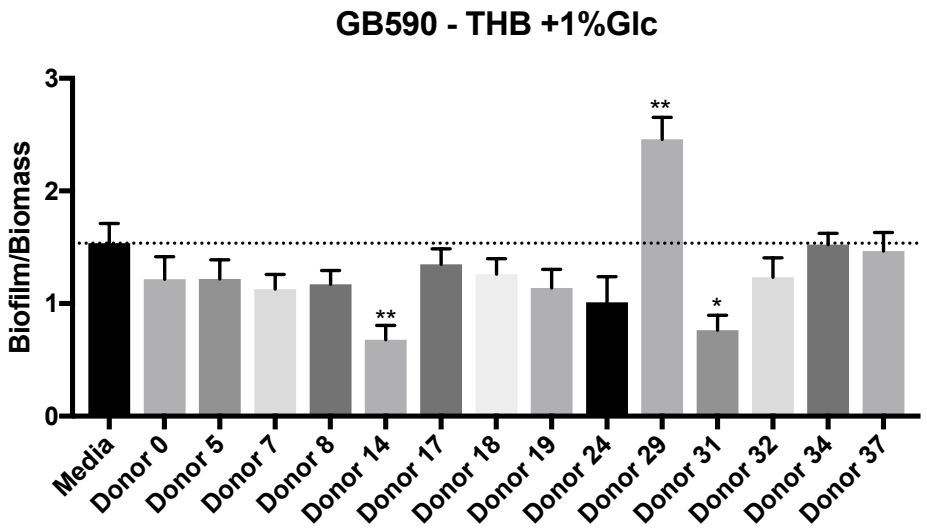


Figure A2.13 Biofilm to biomass ratio of *S. agalactiae* strain GB590 after 24 H of growth in THB + 1% glucose media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data expressed as mean biofilm/biomass ratio measurements (OD_{560}/OD_{600}) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 6.423$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

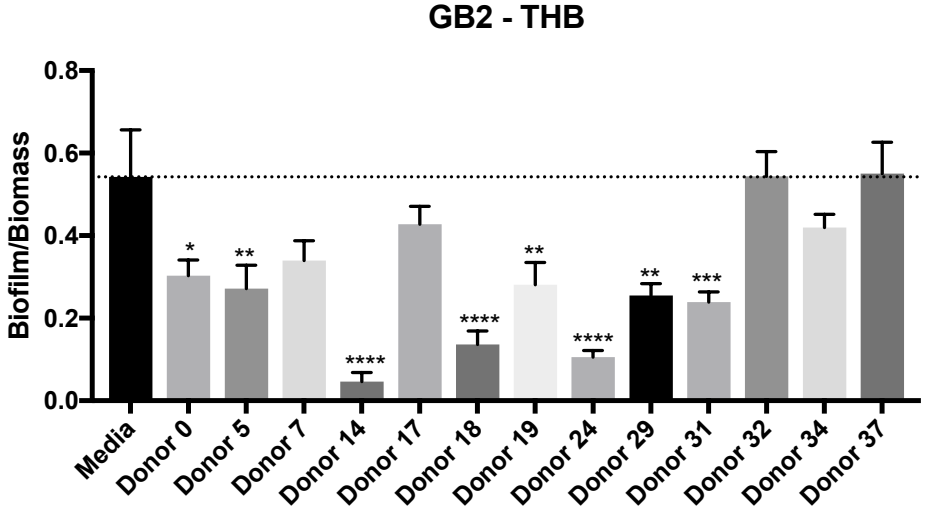


Figure A2.14 Biofilm to biomass ratio for *S. agalactiae* strain GB2 after 24 h of growth in THB media alone or in the presence of ca. 5 mg/mL HMOs from various donors excluding Donor 8. Data expressed as mean biofilm/biomass ratio measurements (OD_{560}/OD_{600}) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 9.692$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs. Results from Donor 8 were determined to be outliers using ROUT ($Q = 1$) and Grubbs (alpha = 0.05) outlier tests.

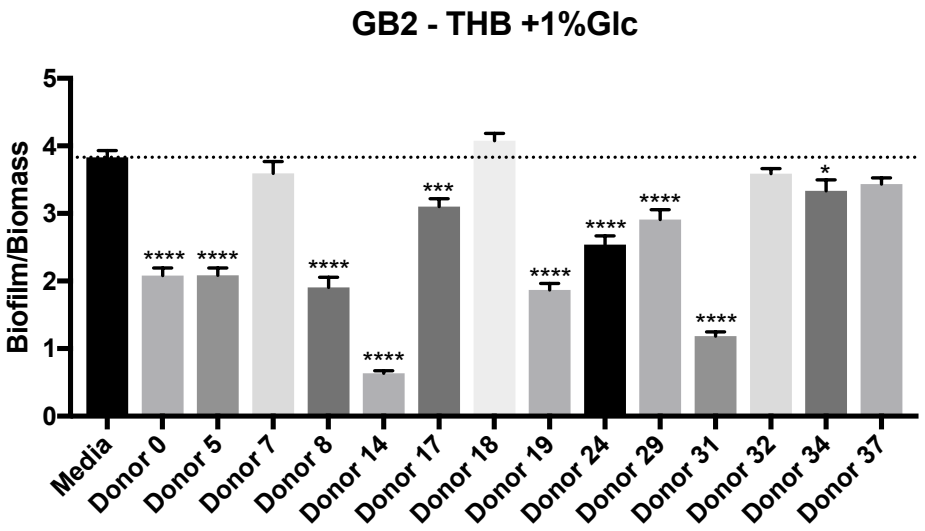


Figure A2.15 Biofilm to biomass ratio of *S. agalactiae* strain GB2 after 24 h of growth in THB + 1% glucose media alone or in the presence of ca. 5 mg/mL HMOs from various donors. Data expressed as mean biofilm/biomass ratio measurements (OD_{560}/OD_{600}) ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 8.55$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs.

CHAPTER IV

Synthesis and Biological Evaluation of a Series of Human Milk Glycoconjugates as Antibacterial Agents against Group B *Streptococcus*

Introduction

Carbohydrates are ubiquitous and play important roles in biological systems, making them attractive subjects in chemical and biological research.[1-3] Carbohydrates influence cellular processes by participating in molecular or cell-cell recognition and adhesion, cellular transport, and cell signaling functions.[2, 4] They appear as monosaccharides, oligosaccharides, and polysaccharides, and as components of peptidoglycans, glycoproteins, nucleic acids, lipopolysaccharides, and glycolipids.

In order to study the mechanisms and structure-activity relationships associated with carbohydrates, they must either be isolated from natural sources, which can be a very tedious process, or synthesized by chemical or enzymatic means.[1, 3, 5] Chemical synthesis of carbohydrates faces two main challenges: controlling the regioselectivity of glycosylation and forming the desired anomeric stereochemistry.[3, 4, 6] Regioselectivity is acheived by differentially protecting the identical functional groups, mainly hydroxyls, present in carbohydrates.[3] Anomeric stereochemistry is largely controlled by the solvent and nature of the C2 protecting group.[2, 3]

Glycosidic linkages are formed by the connection of a donor and an acceptor. A glycosylation begins with activation of the donor's anomeric leaving group (LG) (Figure 4.1). Once the leaving group is activated, it can leave, and an oxocarbenium ion is formed as an intermediate and be stabilized as either a contact ion-pair (CIP) or a solvent-separated ion-pair (SSIP) depending on the polarity of the solvent.[7, 8] SSIP are stabilized by more polar solvents. The

intermediates can be attacked by a nucleophile (the acceptor) at either the top or bottom face resulting in a 1,2-*cis*, α-glycoside or a 1,2-*trans*, β-glycoside, respectively (Figure 4.1). Moreover, the nature and configuration of the protecting groups at C2 also influence the outcome of a glycosylation reaction. For instance, 1,2-*trans* relationships are often favored when C2 is sterically encumbering or the protecting group has the ability to participate in stabilizing the oxocarbenium intermediate.[9] This stabilization occurs by participation from an acyl group at C2 resulting in an acyloxonium intermediate (Figure 4.2).[5] Conversely, a non-participating group at C2, such as an ether, can be used to favor 1,2-*cis* glycosides.[9]

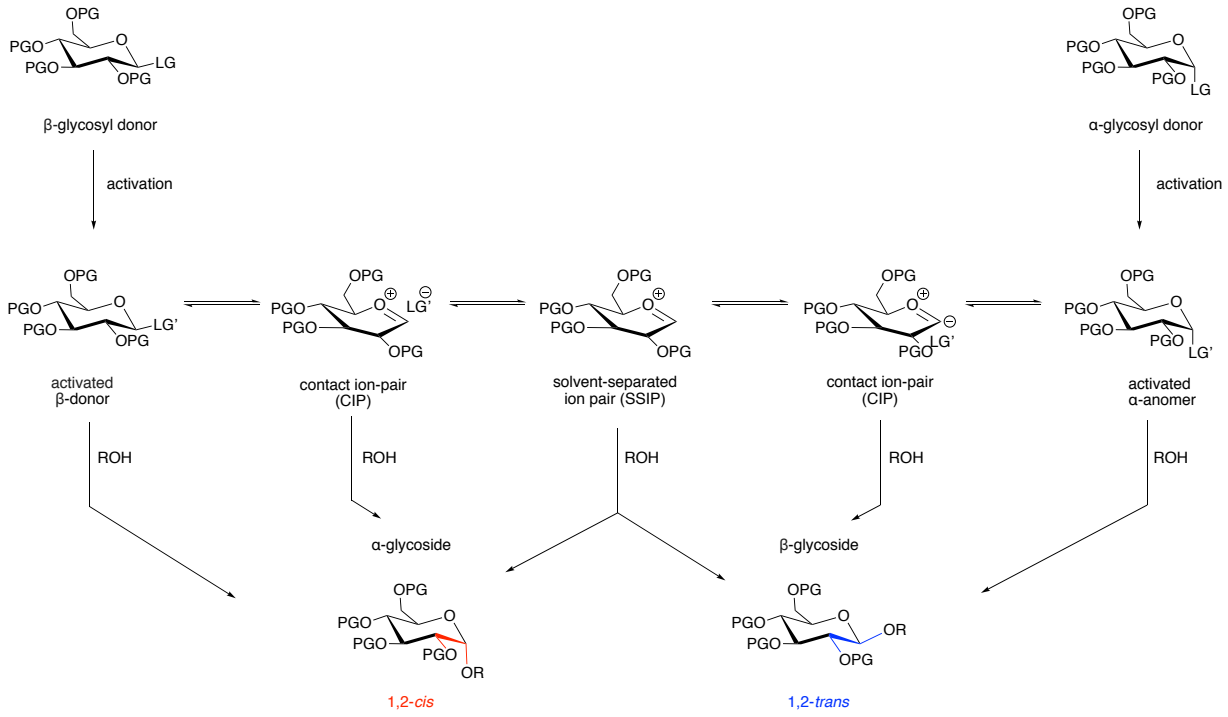


Figure 4. 1 A general glycosylation mechanism. New glycosidic bonds are highlighted in color. LG = leaving group. PG = protecting group.[8]

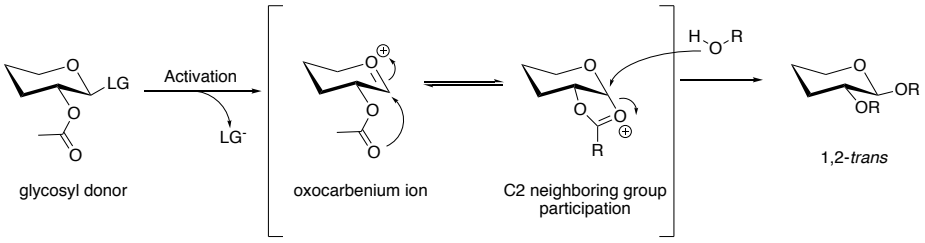


Figure 4. 2 Mechanism of 1,2-trans selective glycosylations due to participation of the C2 substitutent.[5]

A variety of anomeric leaving groups have been developed for use in oligosaccharide synthesis.[3, 5] The leaving groups employed in this study were chosen for their ease of synthetic access and precedence in literature for achieving the desired glycosidic linkages. They include trichloroacetimidates, thiols, fluorides, and phosphites (Figure 4.3).

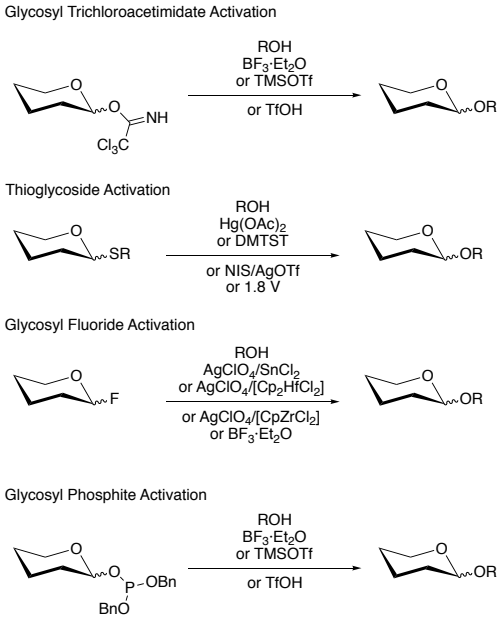


Figure 4. 3 Leaving groups used in this study[2, 5]

Trichloroacetimidates were first employed as leaving groups by Schmidt and coworkers and have since become a popular glycosyl donor due to their ease of synthesis and activation.[5, 10]

Trichloroacetimidates can be activated with catalytic amounts of Lewis acids such as TMSOTf and BF_3•Et_2O and Brønsted acids such as TfOH (Figure 4.3).

Thioglycosides are appealing donors because of their ability to be activated by a broad range of electrophiles, their stability to a considerable amount of intermediate functional group transformations, their easy accessibility, and their capacity to be converted to other leaving groups. Glycoyslations using thioglycosides were first reported in 1973 by Ferrier and coworkers.[11] Metal salts, halonium reagents, organosulfur reagents, and single electron transfer reagents can all be used to activate thioglycosides (Figure 4.3).[12]

Fluoride donors are advantageous over other halides due to their increased stability. Unlike chlorides and bromides, they can withstand purification such as column chromatrography.[13] Mukaiyama first developed the use of fluorides as glycosyl donors in 1981.[14] Fluoride donors were initially activated with a fluorophilic $SnCl_2$-$AgClO_4$ activator, but can also be activated under other conditions including activators with either $SnCl_2$ or $AgClO_4$ ($SnCl_2$-$TrClO_4$ and $SnCl_2$-AgOTf). Lewis acids (BF_3•Et_2O, TMSOTf, $Yb(OTf)_3$, TiF_4, and SnF_4, among many others) and group IV metallocenes (Cp_2ZrCl_2-$AgClO_4$, Cp_2HfCl_2-$AgClO_4$, Cp_2ZrCl_2-$AgBF_4$, and Cp_2HfCl_2-AgOTf) have also proven to be useful activators(Figure 4.3).[14, 15]

Finally, phosphites were independently introduced for use as sialyl donors by Wong and Schmidt.[14, 16, 17] Since then, they have found wide-spread use in sialylations as well as other glycosylations.[18-20] Phosphite donors can be activated with Lewis and Brønsted acids such as BF_3•Et_2O, TMSOTf, and TfOH (Figure 4.3).[5]

Rationale for Synthesis

As detailed in the previous chapters, the carbohydrates in human milk can inhibit the growth and biofilm formation of GBS. The present study tests the hypothesis that the smallest HMOs produced by the mammary gland possess antibacterial activity. This hypothesis was developed based on an initial investigation into the relationship of HMO size and antimicrobial or anti-biofilm properties.

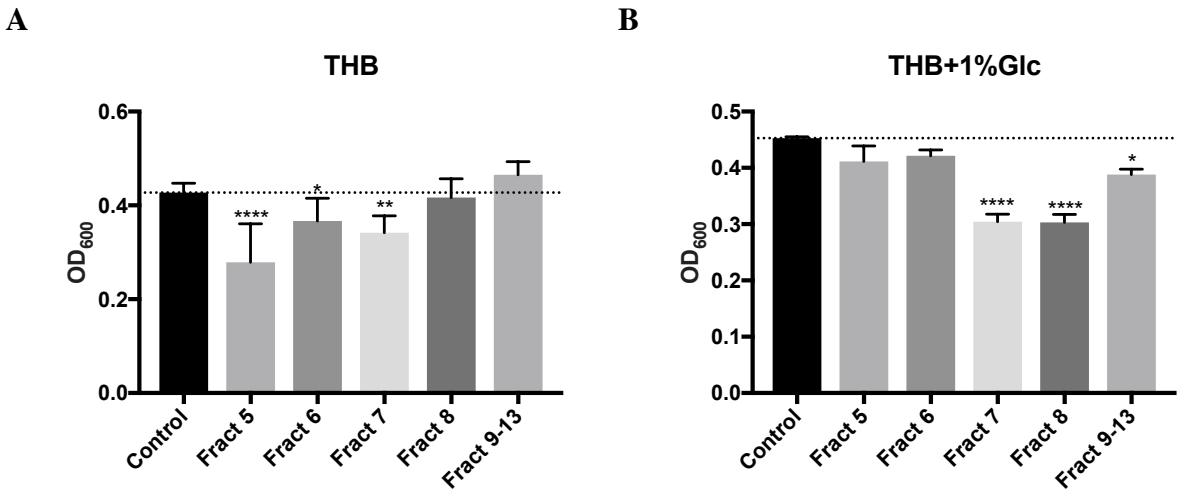


Figure 4. 4 Identification of HMO fractions that inhibit the growth of S. agalactiae strain CNCTC 10/84. The total biomass (growth) after 24 h of growth was compared for (A) THB medium alone. Data represented as the mean biofilm/biomass ratio ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 18.53$ with posthoc Dunnet's multiple comparison test comparing each HMO group against the control sample without HMOs. (B) THB medium supplemented with 1% glucose. Data are expressed as the mean biofilm/biomass ratio ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by oneway ANOVA, $F = 17.89$ with posthoc Dunnet's multiple comparison, compared to media alone.

The native HMOs from donor 42 were fractionated using size exclusion chromatography and organized into five fractions based on LC/MS profiles (Figure A3.1). While HMOs from donor 42 had no significant activity in the initial study, we reasoned fractionating and removing lactose could increase the activity by concentrating active compounds into a few fractions. Each fraction was evaluated for its activity against GBS strain CNCTC 10/84 using a carbohydrate concentration

of 5 mg/mL. The assay revealed that HMOs from Fraction 7 significantly inhibited growth in both THB and THB supplemented with 1% glucose by 20 and 23%, respectively (Figure 4.4). This is encouraging as HMOs from donor 42 had no antimicrobial activity when pooled together (Figure 3.2 and 3.3). Interestingly, the fractions were devoid of anti-biofilm activity (Figure A3.2). Structurally, the HMOs in fraction 7 ranged from trisaccharides to pentasacharides (including HMOs such as 2'/3-FL, LNT, and LNFP-I). Fractions 5 and 6 contained oligosaccharides of 8 units or more, and franctions 8 contained a mixture of disaccharides, trisaccharides, tetrasaccharides. Fractions 9-13 contained both disaccharides and trisaccharides.

We further advanced this hypothesis on the consideration that every HMO is constructed through "polymerization" of a single lactose core (Figure 4.5). At the simplest level, lactose undergoes enzymatic fucosylation or sialylation to generate human milk trisaccharides (See also Chapter 3, Biosynthesis of HMOs, Figure 2.13). These manipulations most commonly occur at the C2/2', C3/3', and C6' positions.

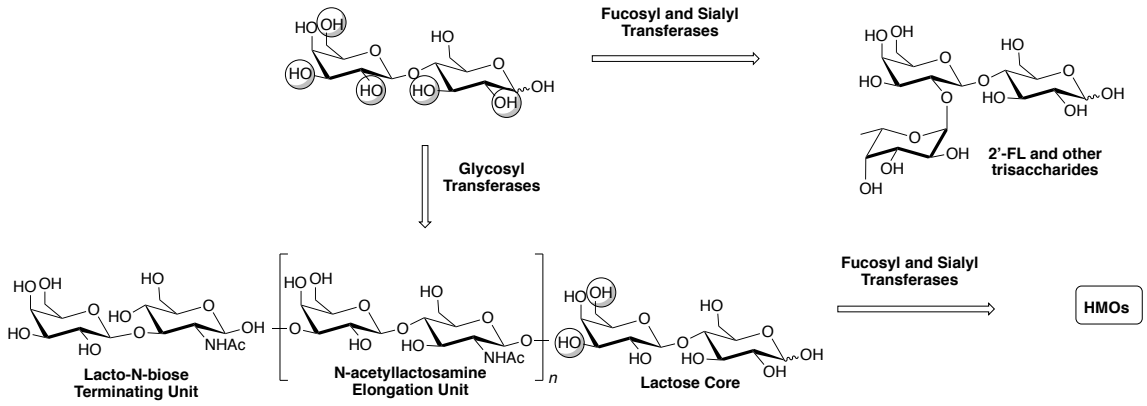


Figure 4. 5 Human Milk Trisaccharide and Oligosaccharide Synthesis

Building upon the initial fractionation study, we synthesized a pool of structurally related

trisaccharides to measure their activity against GBS and to probe the structure-activity relationship of lactose derivatives that are functionalized primarily at C3' (Figure 4.6). In addition to seven synthetic compounds, we included two related, commercially available HMOs in our study. Four of these trisaccharides have been synthesized previously, but none have been tested for their activity against GBS. 3'-Sialyllactose (**2**) has been previously synthesized chemoenzymatically[21-24] and synthetically en route to antigens GM3, Lewis X, and sialyl Lewis X.[25, 26] 3'-Fucosyllactose (**1**)[27] and *epi*-isoglobotriaose (**4**)[28, 29] have been previously prepared chemoenzymatically. Finally, lacto-N-triose (**3**) has been prepared synthetically in a single study.[30]

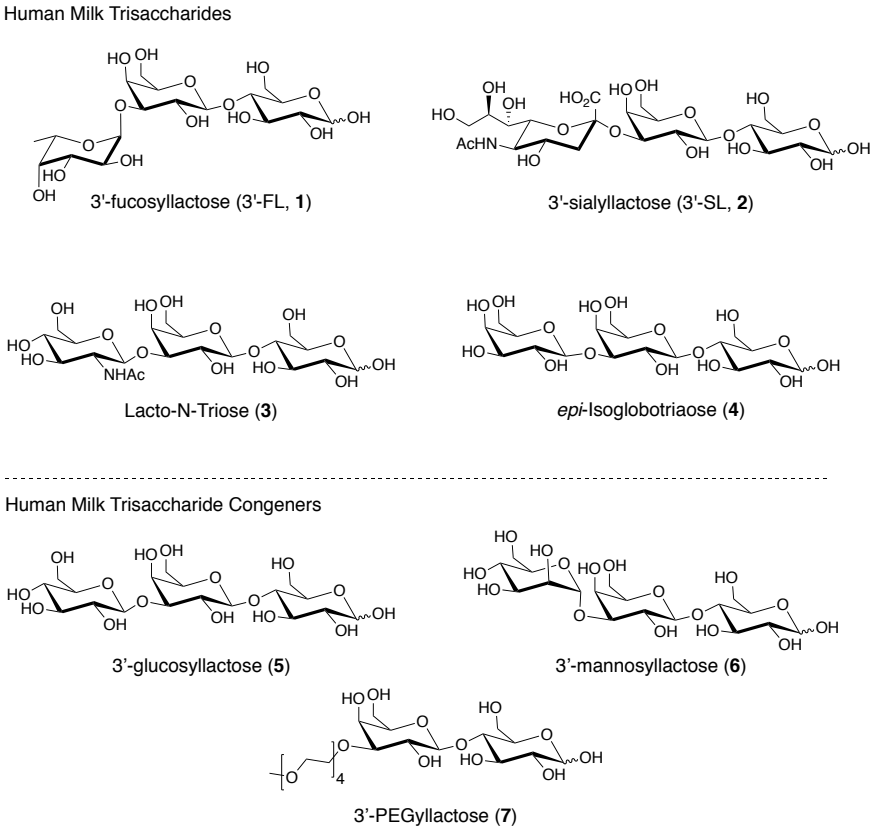


Figure 4. 6 Milk trisaccharides and congeners prepared in this study

We reasoned that a quick, convergent approach to small human milk trisaccharides and

congeners could serve as a place for exploring the individual HMOs or pharmacophores responsible for the antimicrobial and anti-biofilm activity previously observed. Our strategy was to dissect each target compound into two components: 1) a monomeric glycosyl donor and 2) a lactose acceptor that can be prepared on multigram scale in a single pass (Scheme 4.1).

Scheme 4. 1 Synthetic approach to human milk trisaccharides functionalized at the 3' position of lactose.

Synthesis of Human Milk Glycoconjugates

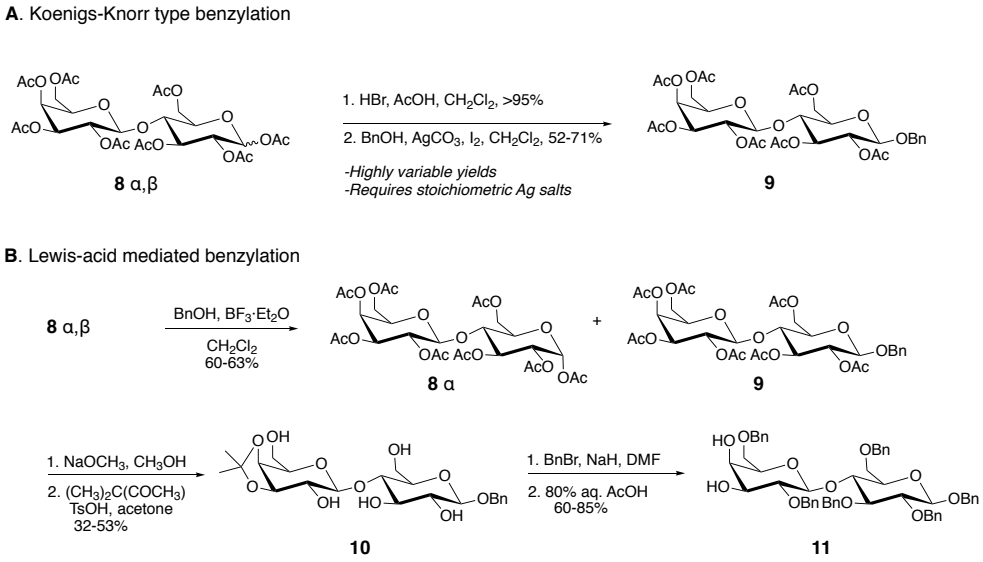
The general structure of one subset of milk trisaccharides features glycosyl residues at C3' of lactose (Figure 4.1). Hence, an initial objective was to prepare a common lactose acceptor with an open site at C3' that could be quickly accessed and used to synthesize seven natural and non-natural derivatives. While the ability to glycosylate the C3'-equatorial hydroxyl group of galactose in the presence of an axial acceptor at C4' is known and we explored this option first,[31] we achieved improved glycosylation yields when using a C4' protected acceptor.

Synthesis of the Lactose Diol Acceptor

An anomeric mixture of **8** was cleanly converted to its α-bromide using HBr/AcOH in CH_2Cl_2 in greater than 95% yield (Scheme 4.2A). Next, a Koenigs-Knorr type reaction with benzyl alcohol gave **9** as a single β-anomer in yields ranging from 52-79%.[31] Unfortunately, this reaction requires stoichiometric amounts of silver salt as a promoter, rendering the sequence unfeasible on a large scale.

To circumvent this problem, we turned to Lewis-acid catalysis (Scheme 4.2B). A BF_3•OEt_2 mediated reaction of **8** with benzyl alcohol gave β-*O*-benzyl lactoside **9** alongside **8**α in 60-63% based on the β-anomer.[32, 33] Presumably, anomeric stability renders **8**α unreactive. Global saponification followed by acetonide formation gives **10**, which can be crystallized from the reaction mixture in moderate yield (32-53%).[33, 34] This reaction also yields the kinetically favored 4',6'-acetonide, which can be recovered and resubjected to the reaction conditions to generate the desired 3',4'-acetonide. Finally, perbenzylation and acetonide hydrolysis gave acceptor **11** in 60-85% yield over two steps.[33, 34] This acceptor can be prepared on multigram scale in a single pass.

Scheme 4. 2 Synthesis of Lactose Diol Acceptor



Synthesis of Monosaccharide Donors

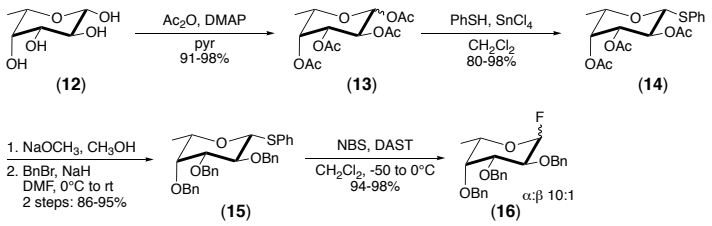
For derivatization of the lactose acceptor **11**, several monosaccharide donors were synthesized. Galactose, glucose, glucosamine, fucose, and neuraminic acid (sialic acid) were chosen for the natural derivatives because these residues are the five monosaccharides that

compose HMOs (Table 2.3). While HMOs do not naturally contain mannose residues, we reasoned that a mannose derivative might have activity due to its prevalence in other human-produced glycans. Additionally, for synthetic ease, we opted to form an α-linked mannoside rather than a β-linked mannoside, which is the linkage naturally found in human glycans. A PEGylated derivative was chosen to test how a carbohydrate mimetic functions in comparison to other derivatives.[35]

Fucosyl fluorides give high α-selectivity and were employed here.[15] Trichloroacetimidates were chosen for glucosamine, galactose, and glucose donors due to their ease of access from the peracetylated derivatives of each monosaccharide.[10] Sialyl phosphite donors have been widely used because of high α-selectivity and improved yields.[19] The thiomannoside was chosen due to commercial availability of the requisite starting material.

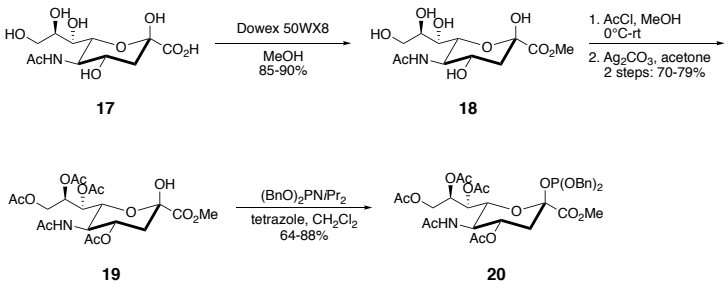
The synthesis of fucosyl fluoride donor **16** started with peracetylation of L-fucose **12** to give **13** in greater than 90% yield (Scheme 4.3).[36] Lewis acid mediated phenyl thioglycoside formation provided thioglycoside **14** in 80-98% yields.[36] Following sodium methoxide saponification of **14**, perbenzylation under standard conditions using sodium hydride and benzyl bromide yields thioglycoside **15** in 88-95% yield over two steps.[36] Finally, thioglycoside **15** is converted to fluoride **16** with good α-selectivity (10:1) and high yields (94-98%) using N-bromosuccinimide (NBS) and diethylaminosulfur trifluoride (DAST).[36] Both fucosyl thioglycoside **15** and fucsoyl fluoride **16** were explored to optimize of fucosylations.

Scheme 4. 3 Synthesis of Fucosyl Fluoride Donor



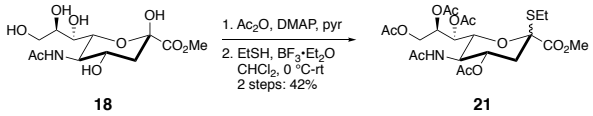
The sialyl phosphite donor **21** can be accessed in 3 steps from N-acetylneuraminic acid **17** (Scheme 4.4).[37] Fisher esterification to form methyl ester **18** (85-90% yield) followed by acetylation and anomeric hydrolysis reveals N-acetylneuraminate **19** in 70-79% yields.[37] Reaction of **19** with dibenzyl N,N-diisopropylphosphoramadite in the presence of tetrazole reveals sialyl phosphite donor **20** in 64-88% yields.[37]

Scheme 4. 4 Synthesis of Sialyl Phosphite Donor



The use of a thioglycoside donor of sialic acid was also explored. Thiol donor **21** can be accessed in two steps from methyl ester **18** by a peracetylation followed by thiol formation using ethane thiol and BF_3•Et_2O in 42% yield over two steps (Scheme 4.5).[38, 39]

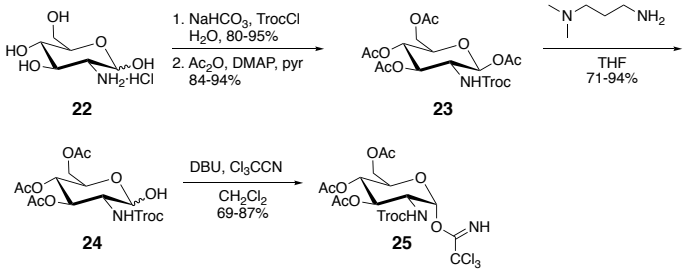
Scheme 4. 5 Synthesis of Sialyl Thiol Donor



18 21

Starting with D-glucosamine hydrochloride **21**, trichloroethyl carbamate (Troc) protection of the amine and peracetylation yields fully protected glucosamine **22** in good yields over 3 steps (84-94%) (Scheme 4.6).[40] Selective cleavage of the anomeric acetate to arrive at intermediate **23** in 71-94% yield was accomplished using dimethylaminopropylamine. Reaction with DBU and trichloroacetonitrile provided 69-87% yield of Schmidt imidate **24**.[40]

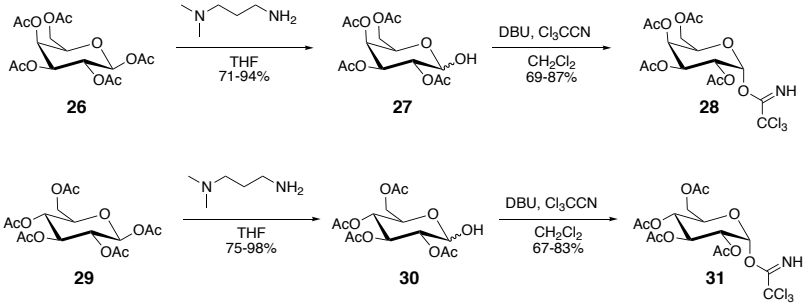
Scheme 4. 6 Synthesis of Glucosamine Imidate Donors



22 23 24 25

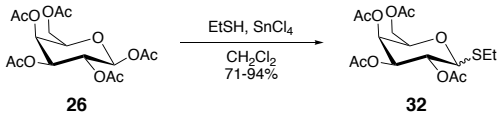
Both galactose and glucose imidate donors were accessed by anomeric deacetylation of galactose pentaacetate **26** or glucose pentaacetate **29** in good yields using dimethylaminopropylamine followed by imididate formation to give galactose donor **28** and glucose donor **31** in 69-87% and 67-83% yields, respectively (Scheme 4.7).[41]

Scheme 4. 7 Synthesis of Galactose and Glucose Imidate Donors



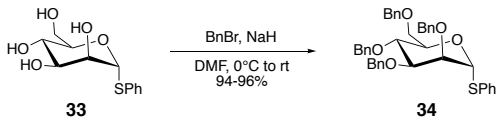
We also explored the use of an ethylthiogalactoside donor **32**, which can be accessed in one step from galactose pentaacetate **26** with $SnCl_4$ and ethane thiol as a mixture of α:β anomers (6:5) in 71-94% yield (Scheme 4.8).[42]

Scheme 4. 8 Synthesis of Thioethyl Galactose Donor



Perbenzylation of commercially available phenyl α-D-thiomannoside **33** gives the thiomannoside donor **34** in one step in greater than 90% yields (Scheme 4.9).[43]

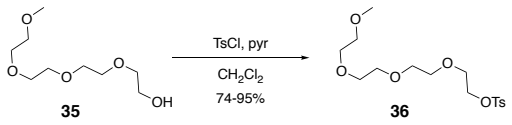
Scheme 4. 9 Synthesis of Thiophenyl Mannose Donor



Tosylation of tetraethyleneglycol monomemthyl ether (PEG-OH) **35** gives **36** which can

be used in an S_N2 reaction with an alkoxide deriviate of the lactose acceptor to form the PEGylated derivative in 74-95% yield (Scheme 4.10).

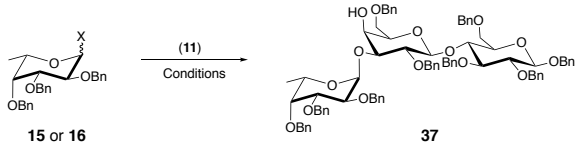
Scheme 4. 10 Synthesis of Tosylated PEG Donor



First Round of Glycosylations using Lactose Diol Acceptor

With the lactose diol acceptor **11** and monosaccharide donors in hand, we were able to explore six glycosylations to arrive at the desired trisaccharides in 20-54% yields. We first explored fucosylation conditions, en route to the trisaccharide 3'-FL (**1**) (Table 4.1). Our plan called for use of a fucosyl fluoride of type **16** (Entries 1-5).[44] Unfortunately, we found this fluoride initially difficult to activate. While excellent selectivity was observed, yields were consistently low. Ultimately, we turned to thioglycoside **16** (Entry 6).[45] Although selectivity suffered and was inconsistent (1.4-2.2:1 α:β), yields increased to 50-65% using the thioglycoside donor, and the resulting anomers were easily separable, giving **37** in reasonable isolated yield.

Table 4. 1 Fucosylation of Lactose Acceptor 11.



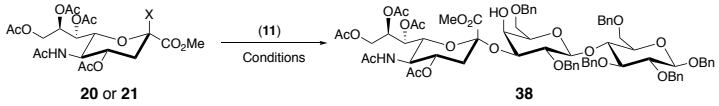
15 or **16** **37**

Entry	Donor (X)	Activator	Yield	α:β[e]
1[a,c]	F	$BF_2 \cdot OEt_2$	22%	α only
2[a,d]	F	$SnCl_2$, $AgClO_4$	16%	α only
3[a,d]	F	Cp_2HfCl_2, AgOTf	20-28%	α only
4[b,d]	F	Cp_2ZrCl_2, AgOTf	5%	α only
5[b,d]	F	Cp_2ZrCl_2 $AgClO_4$	trace	α only
6[a,d]	SPh	NIS/AgOTf	50-65%	1.4:1

[a]solvent = Et_2O/CH_2Cl_2, [b]solvent = THF, [c]temp. = 0°C to r.t., [d]temp. = -40°C to r.t., [e]α:β ratio determined by ^1H NMR

The sialylation of **11** is depicted in Table 4.2. There are a number of useful methods developed to achieve α-sialylation.[46] We initially screened a sialyl chloride (Entry 1) as it could be prepared in a single step from commercially available material. Although we observed exclusive α-selectivity, the yield was only 15%. To improve on this result, we examined a sialyl thioglycoside (Entry 2). While we observed complete α-selectivity, only a mild improvement in yield was achieved (27%). Eventually, we turned to Wong's sialyl phosphite[47] (Entry 3), which provided **38** with exclusive α-selectivity in 43-45% isolated yield.

Table 4. 2 Sialylation of Lactose Acceptor 11.



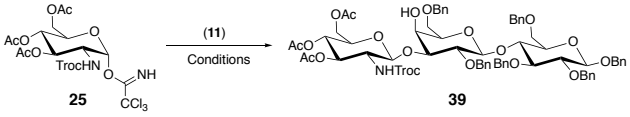
20 or **21** **38**

Entry	Donor (X)	Activator	Yield	α:β[e]
1[a,c]	Cl	Ag_2CO_3, I_2	15%	α only
2[b,d]	SEt	NIS/TfOH	27%	α only
3[b,d]	$OP(OBn)_2$	TMSOTf	43-45%	α only

[a]solvent = CH_2Cl_2, [b]solvent = CH_3CH_2CN, [c]temp. = 0°C to r.t., [d]temp. = -20°C. [e]α:β ratio determined by ^1H NMR.

En route to lacto-N-triose (**3**), we examined the glycosylation of a single glucosamine Schmidt imidate donor **25**, (Table 4.3). Although poor results were observed using BF_3•OEt_2 as a catalyst, TMSOTf provided a clean and moderate yielding glycosylation to give **39** in 35-52% yields.

Table 4. 3 N-acetylglucosylation of Lactose Acceptor 11.



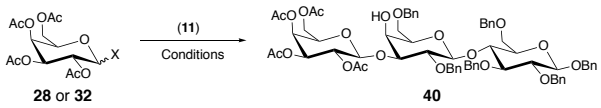
25 **39**

Entry	Activator	Yield	α:β[c]
1[a,b]	$BF_3 \cdot OEt_2$	22-25%	β only
2[a,b]	TMSOTf	35-52%	β only

[a]solvent = CH_2Cl_2, [b]temp = -20°C to r.t. [c]α:β ratio determined by ^1H NMR.

The installation of a galactose residue was accomplished with thioglycoside and imidate galactosyl donors readily prepared from galactose. While the glycosylation with thioglycoside **32** varied in yield (35-52%, Table 4.4, Entry 1), reaction of Schmidt imidate **28** occurred in consistent isolated 51-56% yields and excellent anomeric selectivity to give **40** as a single β-anomer.

Table 4. 4 Galactosylation of Lactose Acceptor 11.

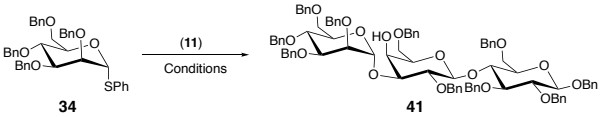


Entry	Donor (X)	Activator	Yield	α:β[e]
1[a,b]	SEt	NIS, AgOTf,	35-52%	β only
2[a,b]	O(C)NHCCl$_3$	NIS/TfOH	51-56%	β only

[a]solvent = CH_2Cl_2, [b]temp = -20°C, [c]α:β ratio determined by ^{1}H NMR

The final glycosylation focused on the addition of a mannose residue. This proceeded in a routine manner using one donor and activation conditions but varying the solvent. Under all conditions, activation of thioglycoside **34** with NBS and AgOTf in the presence of acceptor **11** provided trisaccharide **41** in a completely α-selective manner. When the glycosylation was carried out in diethyl ether, yields of up to 15% were seen (Table 4.5, Entry 1). We postulated this was due the low solubility of the acceptor in diethyl ether. Using a mixed solvent system of 1:1 diethyl ether and dichloromethane resulted in improved yields that ranged from 20-39% (Table 4.5, Entry 2). However, yields further improved to 39-47% when dichloromethane was used as the sole solvent (Table 4.5, Entry 3).

Table 4. 5 Mannosylation of Lactose Acceptor 11.

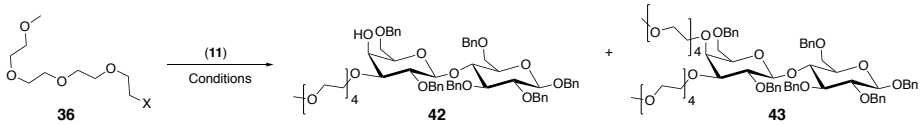


Entry	Solvent	Yield	α:β[c]
1[a,b]	Et_2O	15%	α only
2[a,b]	CH_2Cl_2/Et_2O (1:1)	20-39%	α only
3[a,b]	CH_2Cl_2	39-47%	α only

[a]activator = NBS/AgOTf, [b]temp = -20°C, [c]α:β ratio determined by ^{1}H NMR

In the final study, we recalled polyethylene glycol (PEG) is used to mimic carbohydrates in a number of drugs.[35] Our plan was to add a PEG moiety of similar molecular weight to glycopyranoses. Additionally, we wanted to examine whether 3', 4'-alkoxide salts would show similar regioselective nucleophilicity as their parent alcohols. Initially, we explored the use of a commercially available PEG-Br. Interestingly, regioselective mono-alkylation of **11** with a type **36** PEG-Br proved challenging. When using 1.1 eq. of PEG bromide, the reaction displayed no regioselectivity, resulting in production of both mono- **42** and di-PEGylated lactose **43**, with yields ranging from 19-38% and 10-29%, respectively. Not surprisingly, using excess electrophile gave only di-PEGylated lactose **43** with yields ranging from 30-66%. Exchanging the bromide for a tosylate provided increased yields of **43**. We concluded that while glycosylation of galactosyl type 3,4-diols can occur preferentially at the equatorial acceptor, Williamson ether type alkylation is inherently different. Thus, it appears that a differentiation in nucleophilicity lies only at the alcohol state.

Table 4. 6 PEGylation of Lactose Acceptor 11.



Entry	(X)	Conditions	Yield (42)	Yield (43)
1[b]	Br	NaH, DMF	19%	29%
2[b]	Br	NaH, DMF	38%	10%
3[c]	Br	NaH, TBAI, DMF	-	30-66%
4[d]	OTs	NaH, DMF	-	64-82%

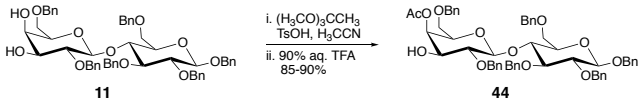
[a]solvent = DMF, [b]temp. = 0°C to r.t., [c]temp. = 80°C, [d]temp. = r.t.

Modified Lactose Acceptor

Gycosylations using the lactose diol acceptor **11** resulted in only moderate yields (up to 56%). As such, we turned our attention to an alternative lactose acceptor with C4' protected as an acetate to determine if yields of the previously described glycosylations could be increased.

The synthesis of the alternative lactose acceptor simply required one modification of the previously described lactose diol acceptor **11**. Staring with lactose diol **11**, an intermediary ortho-ester formation followed by selective opening reveals the 3' lactose acceptor **44** in 85-90% yield.[48, 49]

Scheme 4. 11 Synthesis of Axially Acetylated Lactose Acceptor



Second Round of Glycosylations using Axially Acetylated Lactose Acceptor

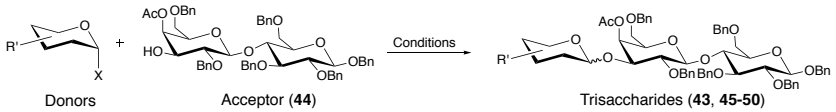
As we approached the glycosylation reactions using the revised lactose acceptor **44**, we opted to use the highest-yielding, most-consistent donors in the initial glycosylation studies with lactose diol **11**. The one exception was the use of fucosyl fluoride instead of the thioglycoside, as it was discovered that an increase in the equivalents of activator ($SnCl_2$-$AgClO_4$) and change in the order of addition resulted in improved yields. Furthermore, we included the installation of a glucose residue to access an additional congener.

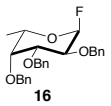
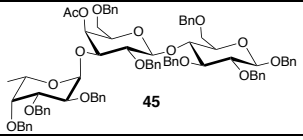
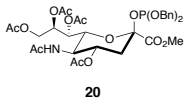
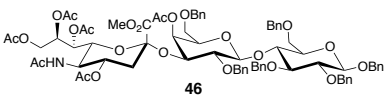
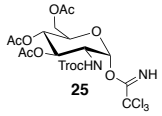
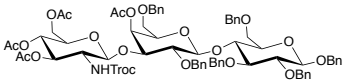
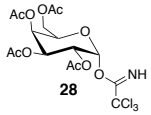
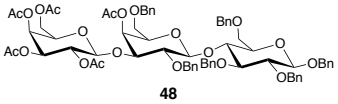
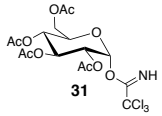
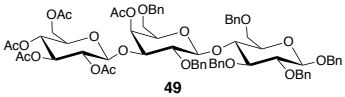
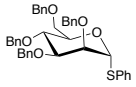
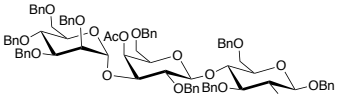
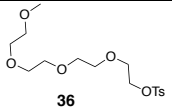
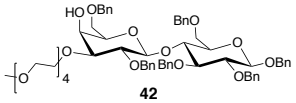
We were pleased to see that the previously optimized glycosylation reactions provided the conditions necessary to produce each compound in high yields as a single anomer. Fucosylation using the fucosyl fluoride **16** was promoted by $SnCl_2$ and $AgClO_4$ to give the protected fucosyl

lactoside **45** in 79% yield with complete α-selectivity. In contrast to this system, sialylation of **44** consistently produced a low yield of the sialylated lactose species. Ultimately, while Wong's phosphite proceeded in a modest 47% yield, only the α-anomer was detected (Table 4.7, Entry 2). Glycosylation with the Schmidt trichloroacetimidates of glucosamine **25**, galactose **28**, and glucose **31** provided the corresponding trisaccharides in excellent yields and anomeric control (Table 4.7, Entries 3, 4 and 5). The final glycosylation focused on a straightforward installation of a mannose residue. Activation of thiophenylmannoside donor **34** with NBS and AgOTf followed by glycosylation occurred in a consistent, high isolated yield (93%), with excellent anomeric selectivity to give the trisaccharide **50** (Table 4.7, Entry 6) as a single α-anomer.

While the previous glycosylations used an excess of donor, an excess of acceptor **44** was used in order to achieve a regioselective mono-PEGylation in 56% yield under Williamson ether synthesis conditions (Table 4.7, Entry 7). While we did observe de-acetylation under these conditions, no diPEGylation was observed.

Table 4. 7 Glycosylations of Lactose Acceptor 44



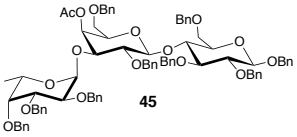
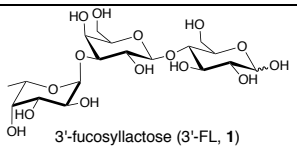
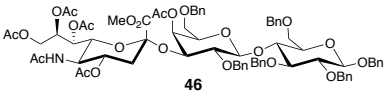
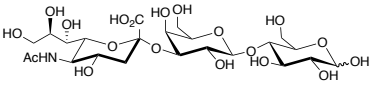
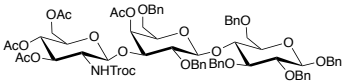
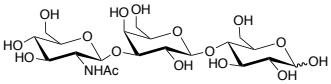
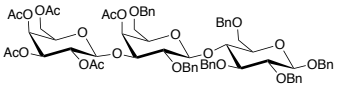
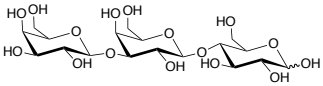
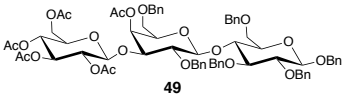
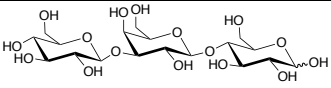
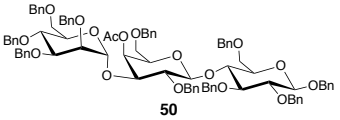
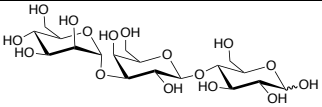
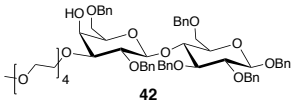
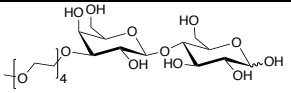
Entry	Donor (x)	Glycosylation conditions	Trisaccharide
1	 16	$SnCl_2$, $AgClO_4$ 1:1Et_2O/CH_2Cl_2, 23°C 79%, α-only	 45
2	 20	TfOH CH_3CH_2CN -30 °C to rt 47%, α-only	 46
3	 25	TfOH CH_2Cl_2 -5 °C 95%, β-only	 47
4	 28	TfOH CH_2Cl_2 -5 °C 92%, β-only	 48
5	 31	TfOH CH_2Cl_2 -5 °C 75%, β -only	 49
6	 34	NIS/AgOTf CH_2Cl_2, -5 °C 93%, α-only	 50
7	 36	NaH, DMF, 0 °C to rt 56%	 42

Deptrotection Strategies for Trisaccharides

When designing the synthesis and selecting protecting groups, we also had in mind the global deprotection strategies required after successful glycosylations. Two main protecting groups were used: acetates and benzyl ethers. Saponification of the acetates with sodium

methoxide followed by hydrogenation of the benzyl ethers using Pearlman's catalyst ($Pd(OH)_2$) yielded the fully deprotected trisaccharides in good yields ranging from 45-93% (Table 4.8). However, the sialyl-containing trisaccharide **46** required a methyl ester for protection of the carboxylic acid, which can be cleaved during the saponification step, using sodium hydroxide in place of sodium methoxide (Table 4.8, Entry 2). The glucosamine-containing trisaccharide **47** incorporated a Troc-protected amine, which required an initial step to remove the Troc group and form the acetimidate in situ using a zinc/lead couple followed by addition of acetic anhydride (Table 4.8, Entry 3).

Table 4. 8 Deprotection Strategies for Trisaccharides

Entry	Trisaccharide	Deptrotection Strategy	Deprotected Glycoconjugate
1	 **45**	1. NaOMe, MeOH 2. $Pd(OH)_2$, H_2, MeOH 80%	 3'-fucosyllactose (3'-FL, **1**)
2	 **46**	1. NaOMe, MeOH 2. $Pd(OH)_2$, H_2, MeOH, then H_2O, 78%	 3'-sialyllactose (3'-SL, **2**)
3	 **47**	1. 10% Zn/Pb, Ac_2O, THF 2. NaOMe, MeOH 3. $Pd(OH)_2$, H_2, MeOH 83%	 lacto-N-triose (3'-GlcNAcLac, **3**)
4	 **48**	1. NaOMe, MeOH 2. $Pd(OH)_2$, H_2, MeOH 90%	 *epi*-Isoglobotriaose (3'-GalLac, **4**)
5	 **49**	1. NaOMe, MeOH 2. $Pd(OH)_2$, H_2, MeOH 93%	 3'-glucosyllactose (3'-GlcLac, **5**)
6	 **50**	1. NaOMe, MeOH 2. $Pd(OH)_2$, H_2, MeOH 45%	 3'-mannosyllactose (3'-ManLac, **6**)
7	 **42**	$Pd(OH)_2$, H_2, MeOH, 79%	 3'-PEGyllactose (3'-PEGLac, **7**)

We have developed a scalable synthesis of a lactose acceptor that can be used in gram-scale glycosylations to produce human milk trisaccharides and congeners. The general strategy of glycosylating C3-equatorial hydroxyl groups in the presence of an axial hydroxyl group at C4 can be successful, but we achieved better yields when C4 was protected. Additionally, this regioselectivity breaks down when the hydroxyl groups are converted to their corresponding alkoxide salts. However, previous research with stannylene acetals has shown that mono-alkylation of diols is achievable.[50] Specifically, numerous groups have shown high regioselectivity of the C3, C4-galactose type diols to give alkylation at C3, which is postulated to be due to the dimeric or polymeric intermediates of stannylene acetals that position one oxygen for nucleophilicity over the other because it is only coordinated to one tin (Figure 4.7). [51-54]

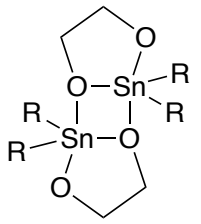


Figure 4. 7 Dimeric structure of stannylene acetals[54]

Evaluation of Biological Activity of Trisaccharides and PEG-Congener

We evaluated each compound (Figure 4.6) and two commercially available HMOs for antibacterial and anti-biofilm activity against three strains of GBS (GB590, GB2, CNCTC 10/84) representing the three serotypes (III, Ia, and V) responsible for greater than 85% of neonatal infection. 2'-FL and 6'-SL (Figure 4.8) were attained from commercial sources to serve as constitutional isomers for two of the trisaccharides synthesized above (3'-FL and 3'-SL). We

reasoned that testing these two constitutional isomers, which are also naturally found in human milk, would guide our understanding of the significance of the branching and substitution pattern of small HMOs.

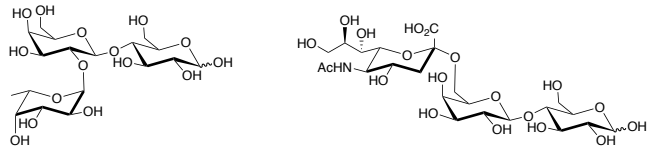


Figure 4. 8 Additional Trisaccharides

The effect of each trisaccharide on the three GBS strains was evaluated at 24 h in THB and THB supplemented with 1% glucose using the previously described plate-based biofilm assay. All assays were performed using a glycoconjugate concentration of 5 mg/mL as this value approximates low physiological concentrations; HMOs are typically found in breast milk at 5–20 mg/mL.[55]

First, we determined antimicrobial by comparing the biomass of bacteria grown in the presence of glycoconjugates to a control (bacteria grown in unsupplemented media). Several compounds were found to significantly inhibit bacterial growth for GBS in at least one set of growth conditions ($p \leq 0.05$ by one-way ANOVA with posthoc Dunnett's multiple comparison test). The results are presented as the percent growth of the control ± SEM of four independent experiments each with three technical replicates. In these studies, 3'-FL (**1**) and 3'-GalLac (**4**) showed interesting activity. 3'-FL (**1**) inhibited the growth of CNCTC 10/84 (THB supplemented with 1% glucose) and GB590 (THB) by 16% and 17%, respectively (Figures 4.9 and 4.10). 3'-GalLac (**4**) showed greater inhibitory activity across each strain with values ranging from 17%

against 10/84 (THB supplemented with 1% glucose), 41% against GB590 (THB), and values as high as 24% against GB2 (THB supplemented with 1% glucose) (Figures 4.9, 4.10, 4.11). As observed in our previous studies, these values varied based on growth conditions.

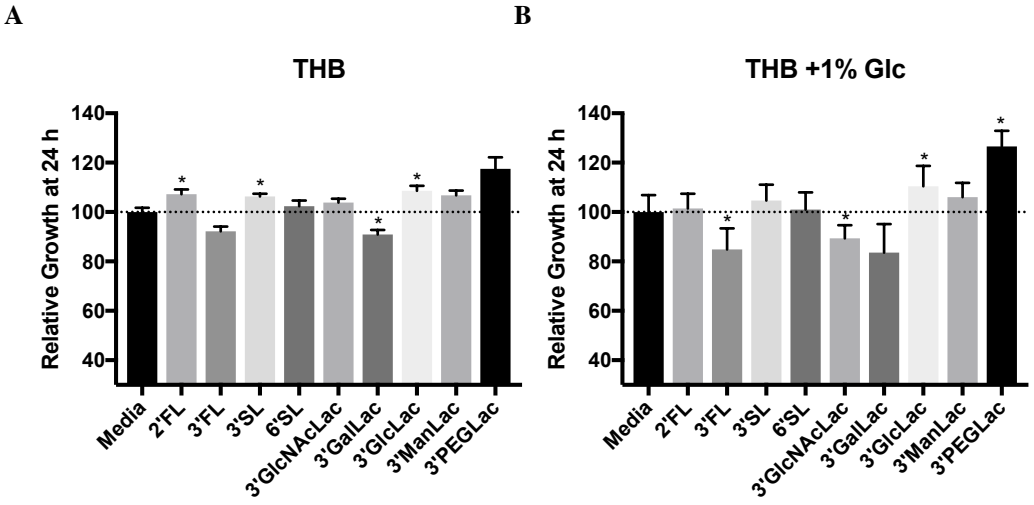


Figure 4. 9 Antimicrobial assays of S. agalactiae strain CNCTC 10/84 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * represents $p < 0.05$ by one-way ANOVA, F = (A) 8.291 and (B) 9.791 with posthoc Dunnet's multiple comparison test comparing each sample against the control.

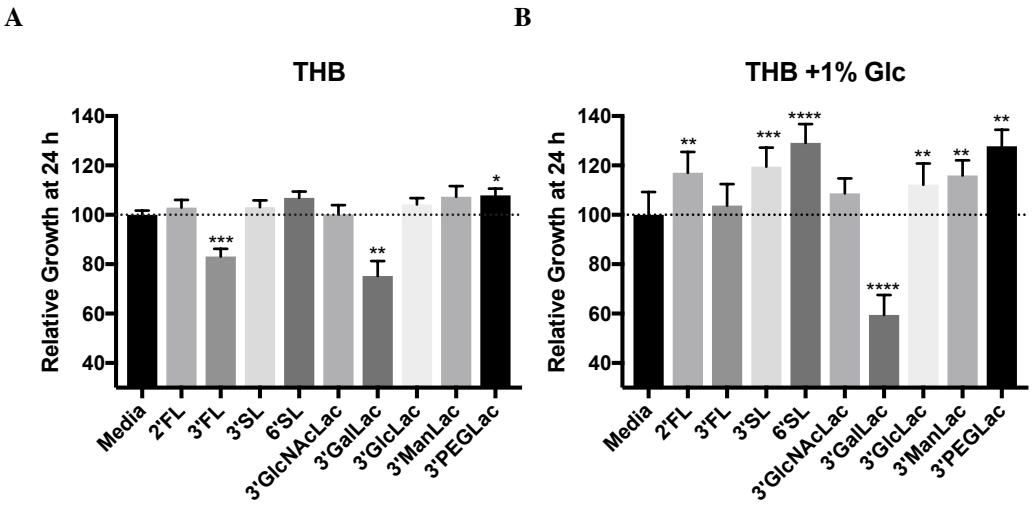


Figure 4. 10 Antimicrobial assays of S. agalactiae strain GB590 at 24 h was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 17.69 and (B) 32.48 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

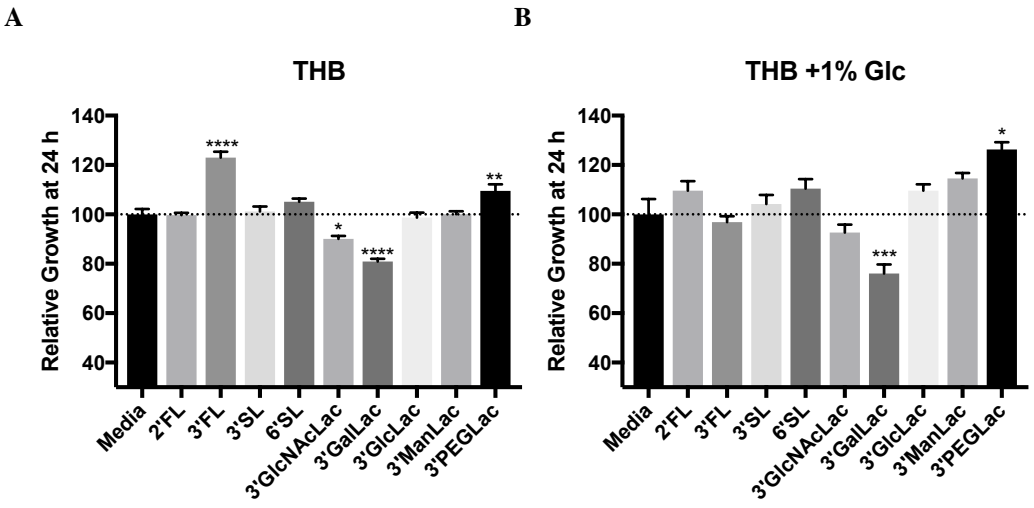


Figure 4. 11 Antimicrobial assays of *S. agalactiae* strain GB2 at 24 h was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *and* **** $p < 0.0001$ by one-way ANOVA, F = (A) 56.29 and (B) 29.37 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

To determine changes in biofilm production, we compared biofilm/biomass ratios of bacteria grown in the presence of HMOs to the control (media). This ratio accounts for antimicrobial activity and permits analysis of changes in biofilm production relative to the number of bacterial cells. The data represents the mean percent biofilm/biomass (OD_{560}/OD_{600}) of the control ± SEM of four separate experiments, each with three technical replicates. Using this standard, three compounds significantly reduced biofilm formation in at least one GBS strain. Interestingly, the fucosylated derivatives (2'-FL, **51** and 3'-FL, **1**) exhibited the greatest biofilm inhibition. 2'-FL (**51**) decreased the amount of biofilm produced relative to growth for CNCTC 10/84 grown in THB by 25% (Figure 4.12) and for GB590 grown in THB supplemented with 1% glucose by 19% (Figure 4.13). Additionally, 3'-FL (**1**) also showed inhibition of biofilm production for GB2 in THB supplemented with 1% glucose (Figure 4.14). Of particular interest, 3'PEGLac (**7**) showed anti-biofilm activity across all strains ranging 14% to 27%, though none of

these values were deemed significant. This is likely due to variation in biofilm measurements after plate workup.

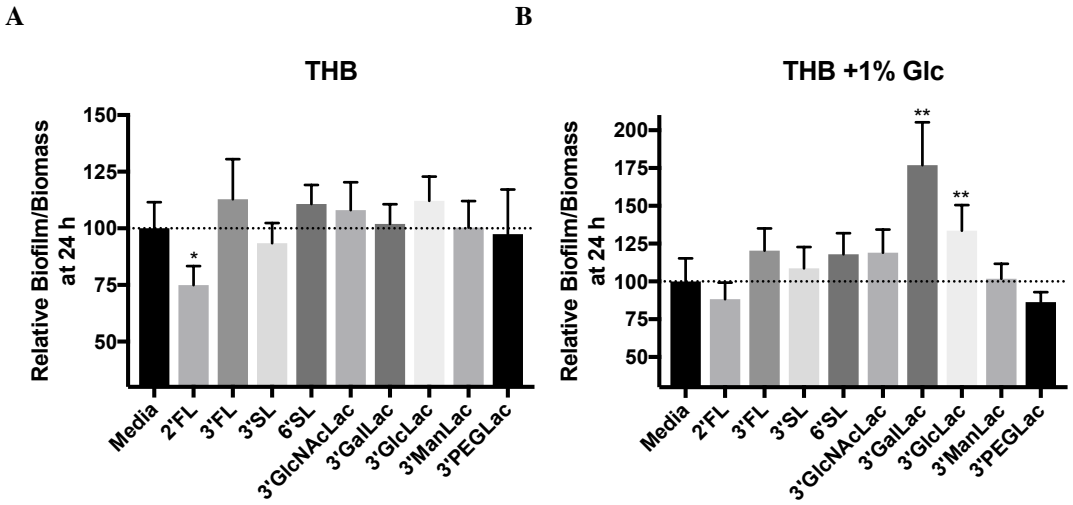


Figure 4. 12 Biofilm/biomass ratios for CNCTC 10/84 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 1.774 and (B) 10.1 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

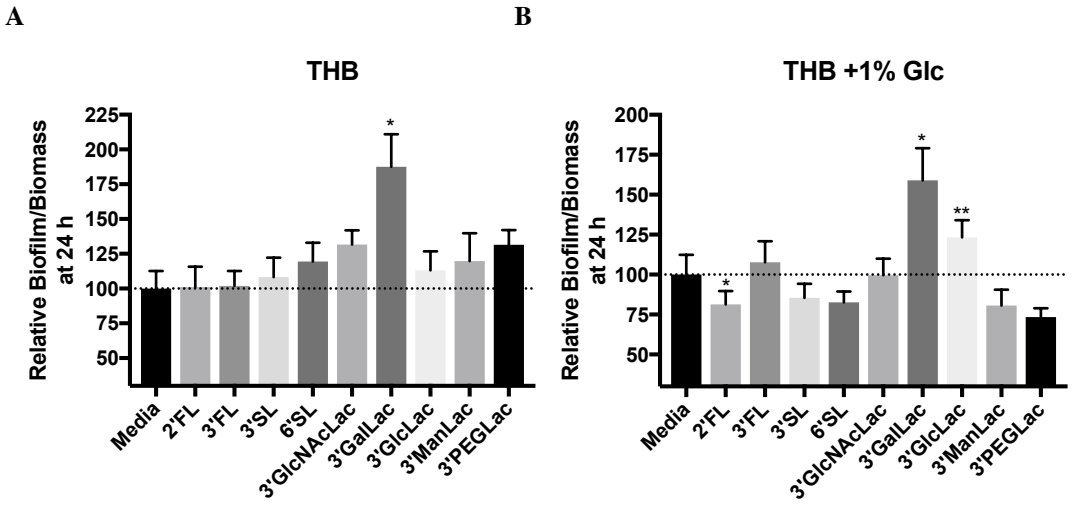


Figure 4. 13 Biofilm/biomass ratios for GB590 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 7.16 and (B) 10.13 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

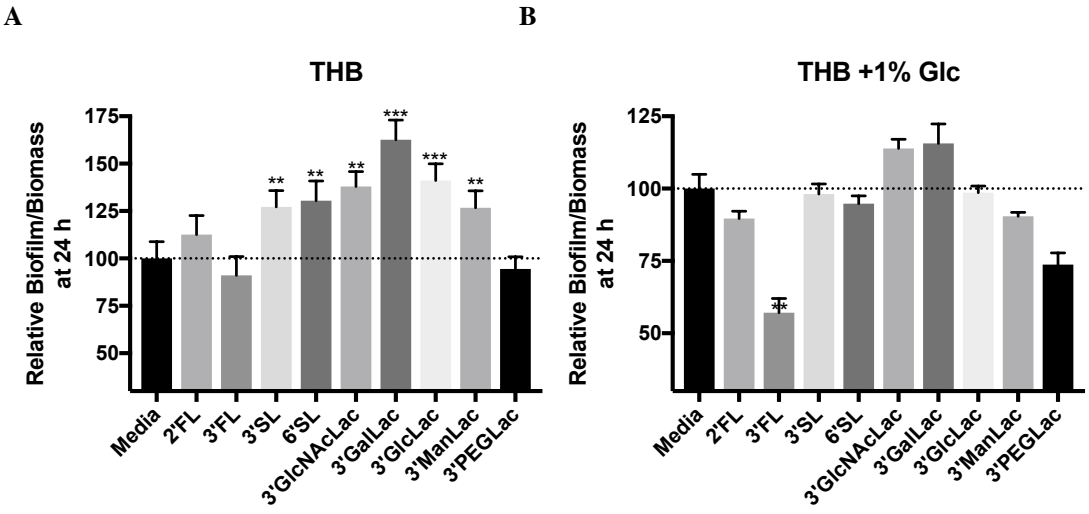


Figure 4. 14 Biofilm/biomass ratios for GB2 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 16.69 and (B) 23.06 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

We also tested the antimicrobial and anti-biofilm activity of monosaccharides, lactose, select oligosaccharides, and other carbohydrate derivatives that are currently used as additives to infant formula. The structures of the compounds assayed are shown in Figure 4.15. The monosaccharides evaluated are the individual constituents of each of the trisaccharides synthesized above. We also wanted to compare the activity of lactose to ensure that the activity seen from the individual trisaccharides and pooled HMOs tested in Chapter 3 was indeed due to the oligosaccharides since lactose has such a high abundance in milk and was not removed from human samples. Further, we chose galacto-oligosacchrides (GOS), fructo-oligosaccharides (FOS), sucrose, lactulose, lactitol, xylans, maltodextrin, and laminarin because they are compounds that are or have been supplemented in infant formula.[56] Maltose and cellobiose are glucose disaccharides that differ in their glycosidic linkage and are used as food additives. Finally, dextran sulfate and colominic acid are charged oligosaccharides consisting of sulfated glucose and sialic acid monomers, respectively. The same plate-based assay as above was used to evaluate the

activity of these compounds.

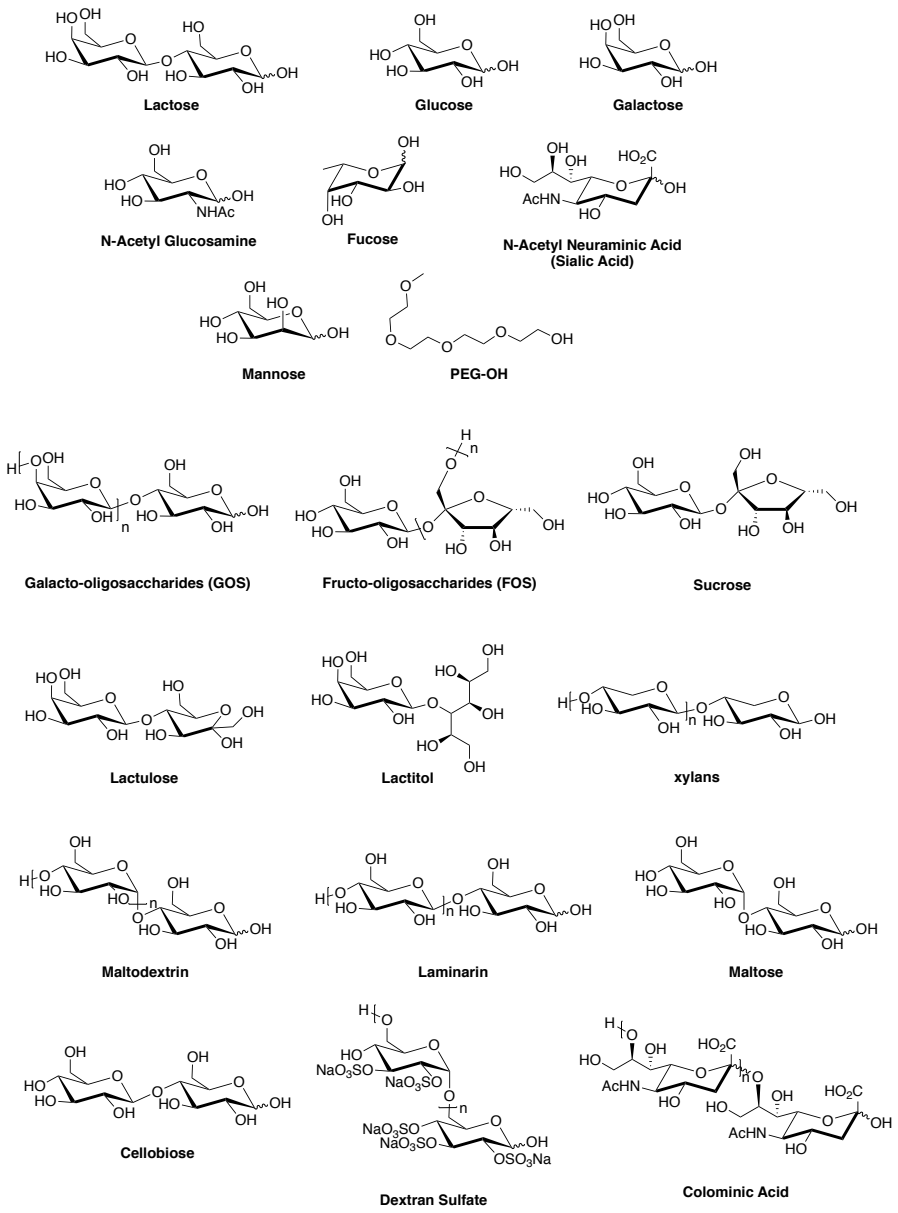


Figure 4. 15 Structures of Additional Carbohydrates Tested against GBS

Neither lactose nor any of the monosaccharides significantly decreased the growth any of

the three GBS strains tested in either growth condition (THB or THB supplemented with 1% glucose). Instead, each of these carbohydrates significantly increased the growth in at least one strain in one or both growth condition (Figure 4.16, 4.16, and 4.18). Growth increased up to 26% compared to the control sample grown in media alone. GBS has multiple genes for carbohydrate transport and metabolism that are differentially expressed according to growth conditions.[57, 58] It is likely that these specific strains have a transcriptome with genes for metabolizing these or similar carbohydrates, and the increase in growth of GBS is due to metabolism of these compounds after the upregulation of these genes.

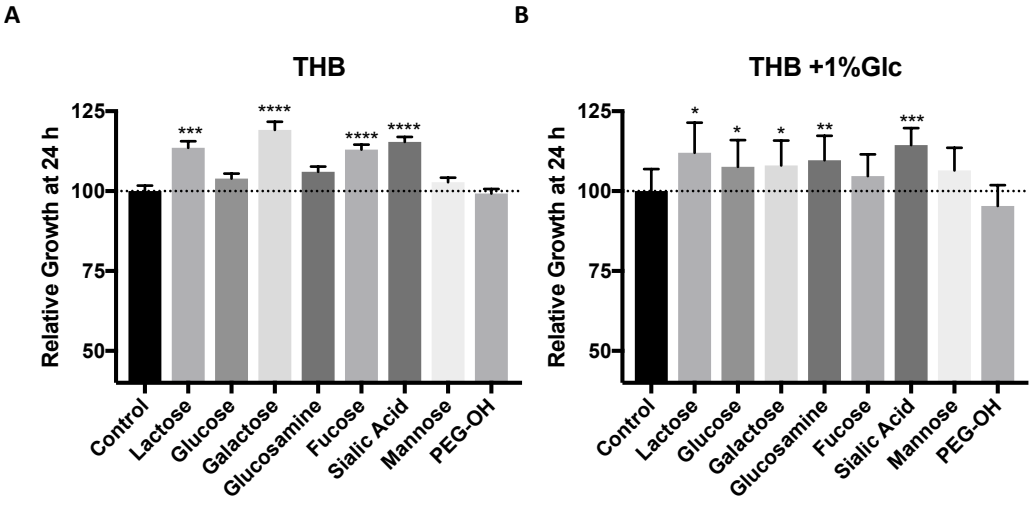


Figure 4. 16 Antimicrobial assays of S. agalactiae strain CNCTC 10/84 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 28.88 and (B) 7.261 with posthoc Dunnet's multiple comparison test comparing each sample against the control.

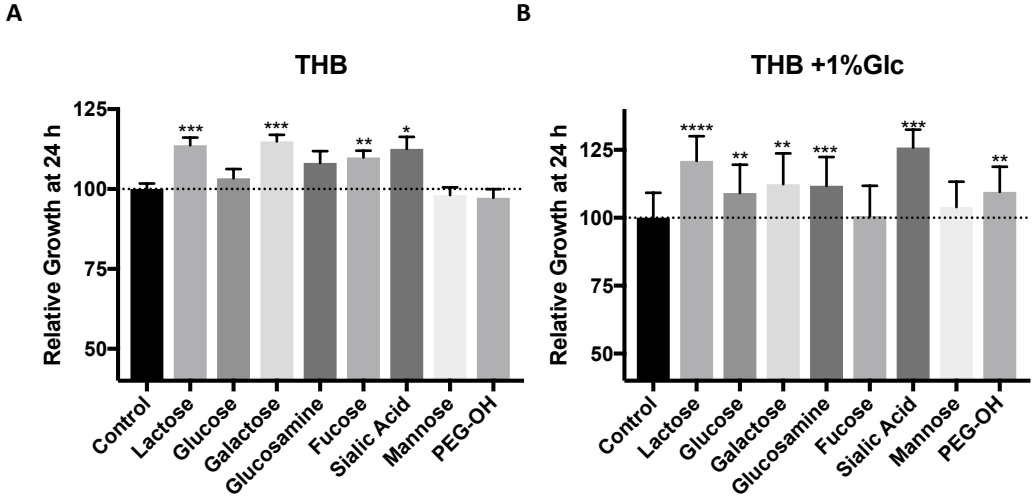


Figure 4. 17 Antimicrobial assays of S. agalactiae strain GB590 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 9.95 and (B) 11.84 with posthoc Dunnet's multiple comparison test comparing each sample against the control

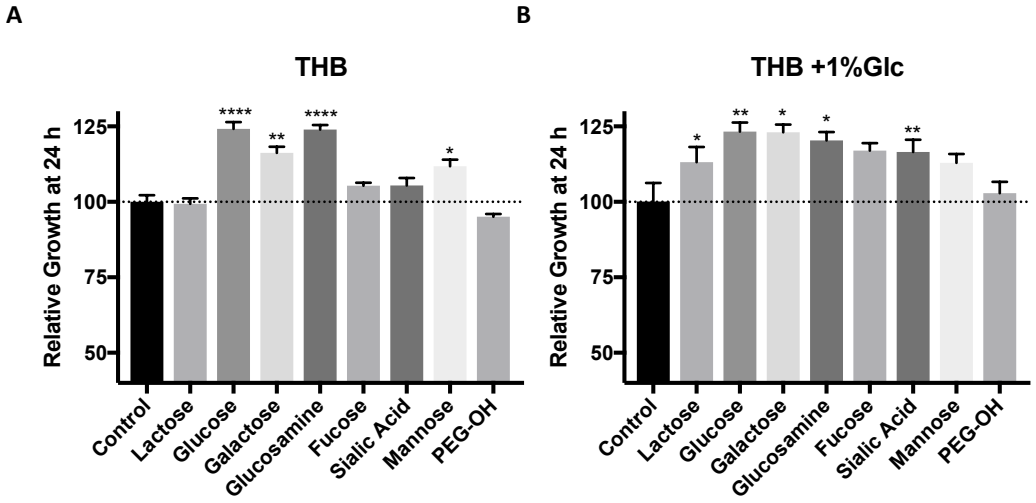


Figure 4. 18 Antimicrobial assays of S. agalactiae strain GB2 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 32.23 and (B) 9.674 with posthoc Dunnet's multiple comparison test comparing each sample against the control.

Lactose and the monosaccharides had little impact on the biofilm as determined by the biofilm to biomass ratio. Only sialic acid significantly reduced the biofilm production in all three strains in at least one growth condition (Figure 4.19, 4.20, and 4.21) with percent reduction ranging from 15-30%. The ability of sialic acid to affect the production of biofilm could potentially be due to the presence of sialic acid in the capsule of GBS. In all GBS strains, sialic acid is a terminal residue in the capsular polysaccharide repeating unit and the capsule is associated with GBS virulence, such as biofilm formation. Perhaps there is a feedback loop that causes GBS to down regulate its biofilm production with excess sialic acid in the media.

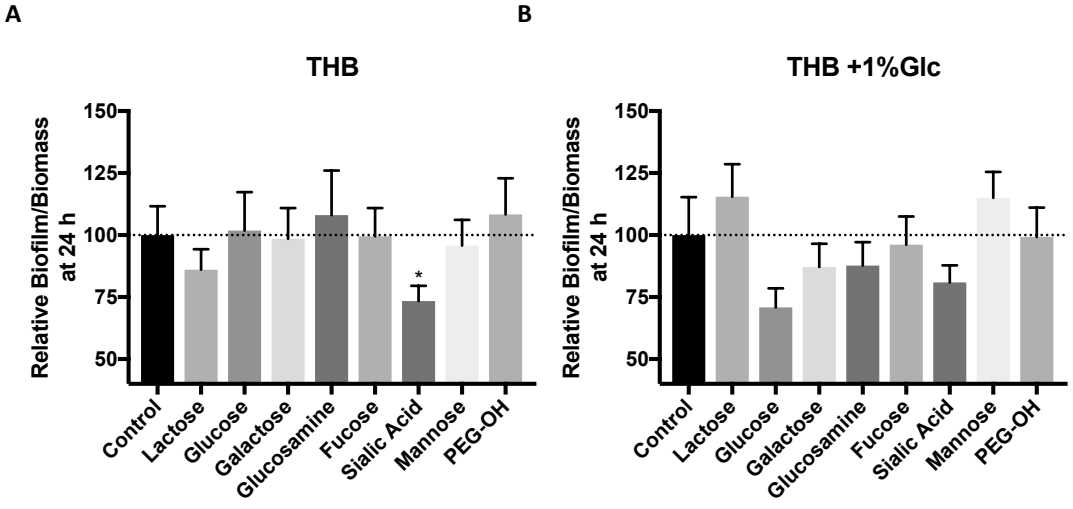


Figure 4. 19 Biofilm/biomass ratios for CNCTC 10/84 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * represents $p < 0.05$ by one-way ANOVA, F = (A) 2.551 and (B) 6.979 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

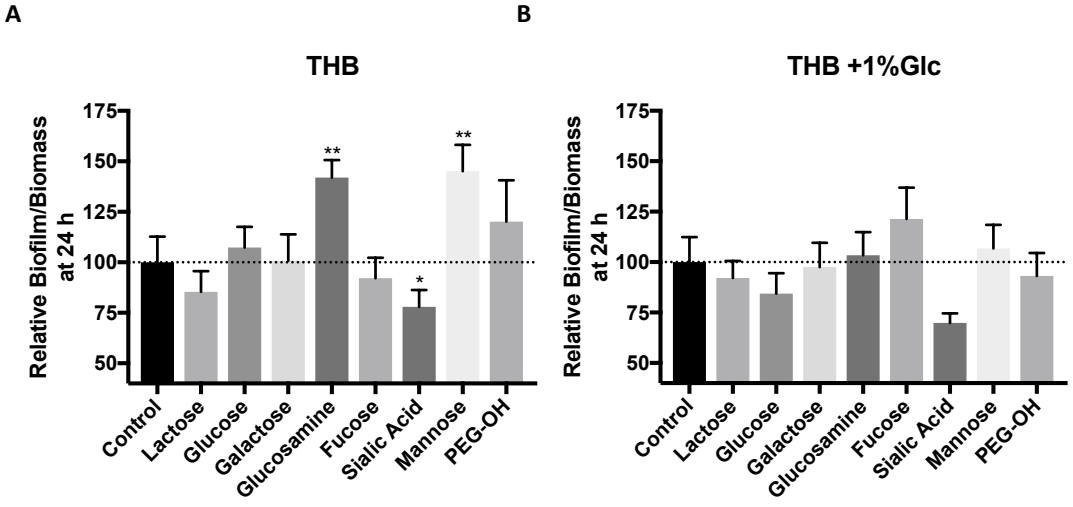


Figure 4. 20 Biofilm/biomass ratios for GB590 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 13.43 and (B) 9.306 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

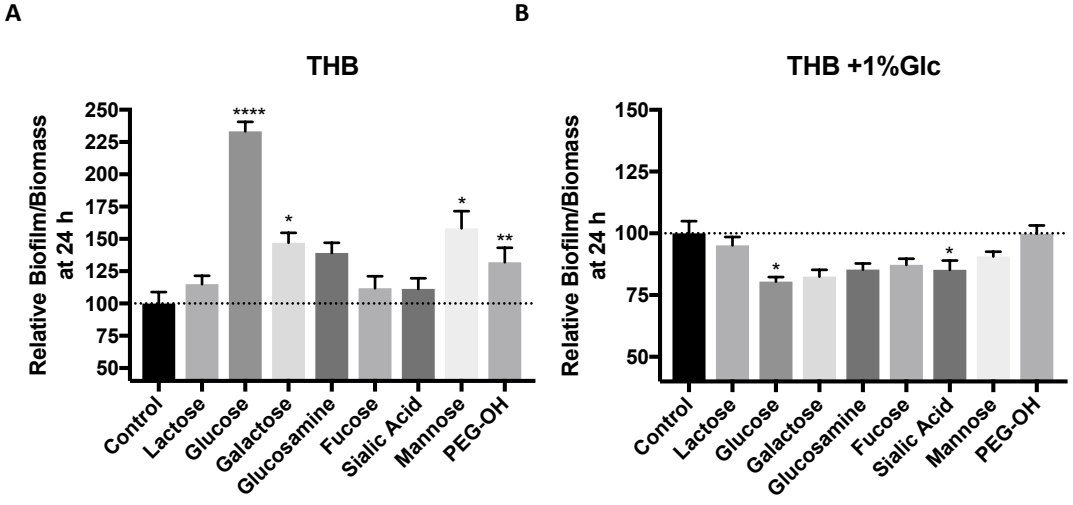


Figure 4. 21 Biofilm/biomass ratios for GB2 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 28.85 and (B) 6.997 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

Several commercially available oligosaccharides were able to significantly inhibit the growth of GBS. The greatest growth inhibition across all strains was achieved with colominic acid, an oligosaccharide composed of sialic acid monomers. In the presence of colominic acid, the reduction of growth of GBS ranged from 9-40% (Figure 4.22, 4.23, and 4.24). Sialic acid is present in the capsule of all GBS strains in an α-2,3 linkage with galactose. Colominic acid has α-2,8 linkages. The presence of sialic acid may trigger the bacterial use of this carbohydrate for capsule synthesis, but it cannot be utilized due to the linkages in the polymer. In strain CNCTC 10/84, xylans, laminarin, and dextran sulfate significantly inhibited the growth in both THB and THB supplemented with 1% glucose. Growth reduction of these oligosaccharides ranged from 8-35%. Maltodextrin was also able to inhibit the growth in strain CNCTC 10/84 in THB supplemented with 1% glucose by 35%.

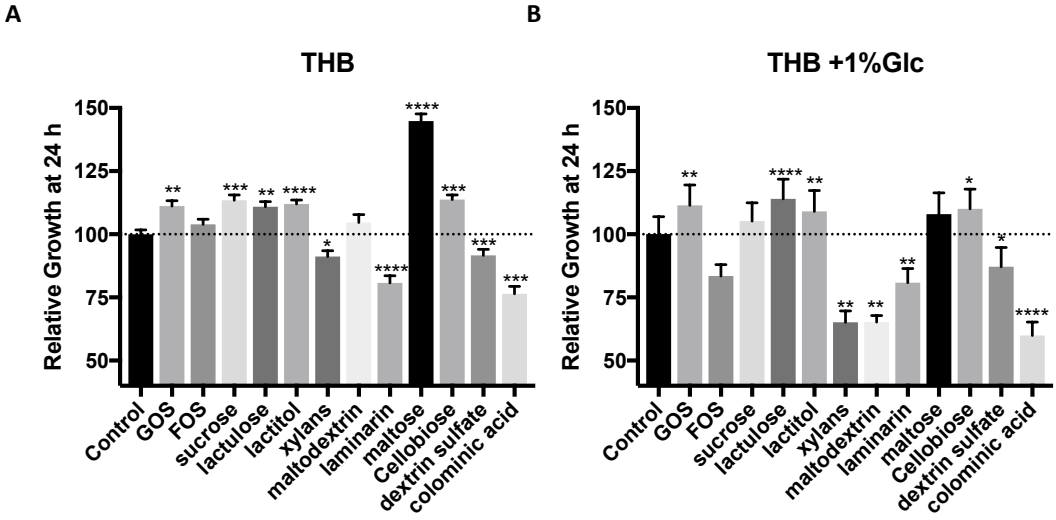


Figure 4. 22 Antimicrobial assays of S. agalactiae strain CNCTC 10/84 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 79.36 and (B) 38.26 with posthoc Dunnet's multiple comparison test comparing each sample against the control.

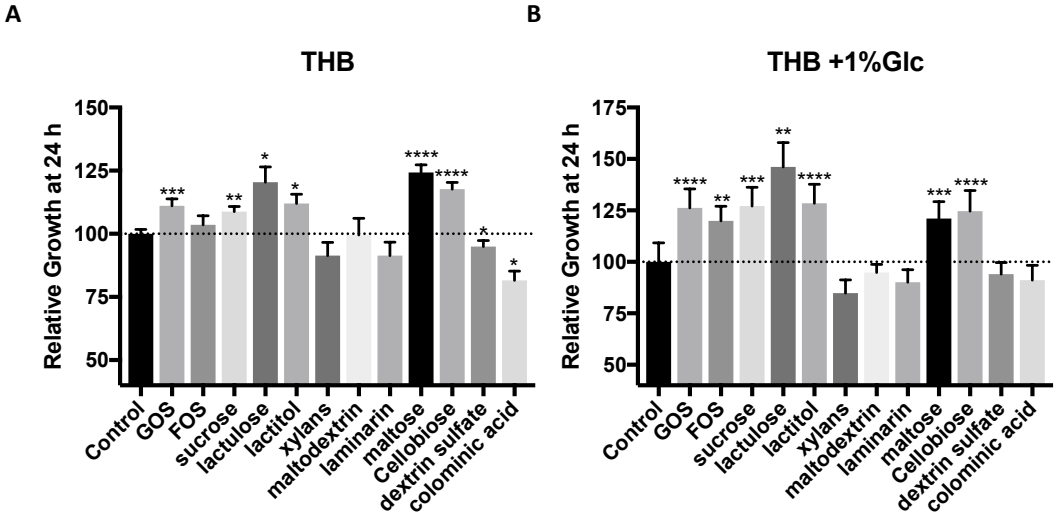


Figure 4. 23 Antimicrobial assays of S. agalactiae strain GB590 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 19.36 and (B) 18.31 with posthoc Dunnet's multiple comparison test comparing each sample against the control.

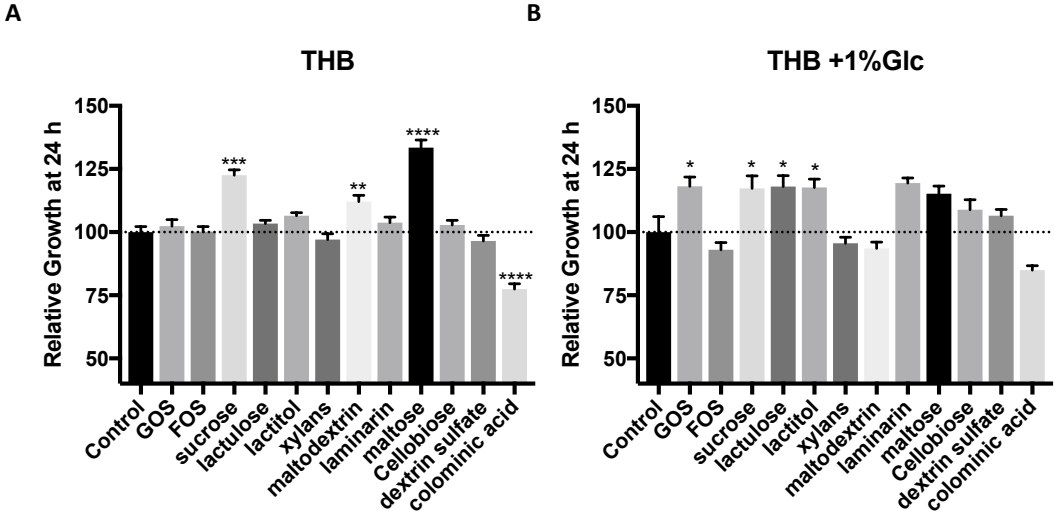


Figure 4. 24 Antimicrobial assays of *S. agalactiae* strain GB2 at 24 h was compared for (A) THB media of (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 44.07 and (B) 16.71 with posthoc Dunnet's multiple comparison test comparing each sample against the control.

While many of the oligosaccharides significantly increased the biofilm produced relative to the biomass, GOS and dextrin sulfate were able to significantly decrease the biofilm/biomass in separate strains in THB supplemented with 1% glucose. GOS exhibited 24% biofilm reduction in GB590, and dextrin sulfate showed a biofilm reduction of 23% compared to the control in GB2 (Figures 4.25, 4.26, 4.27).

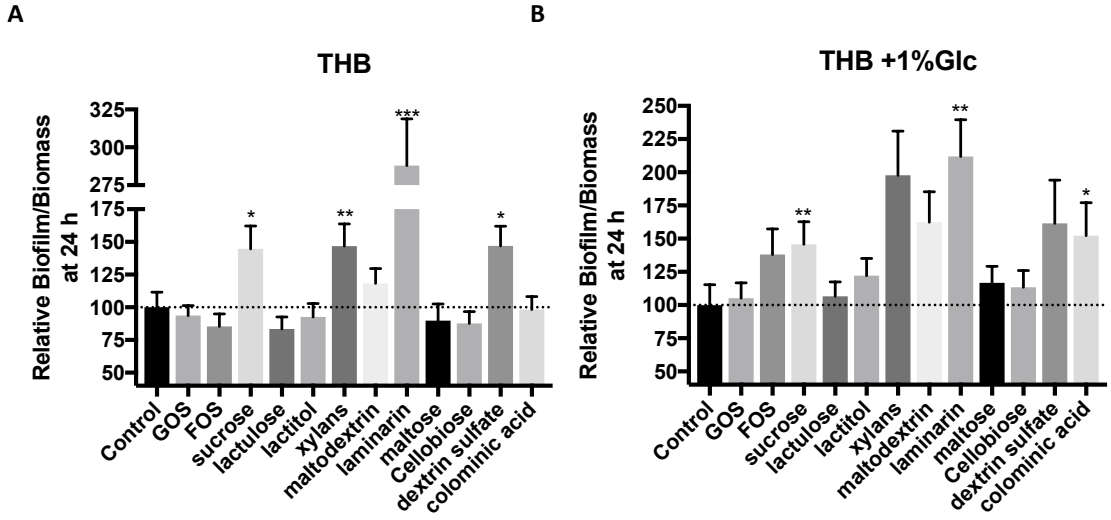


Figure 4. 25 Biofilm/biomass ratios for CNCTC 10/84 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 20.57 and (B) 5.046 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

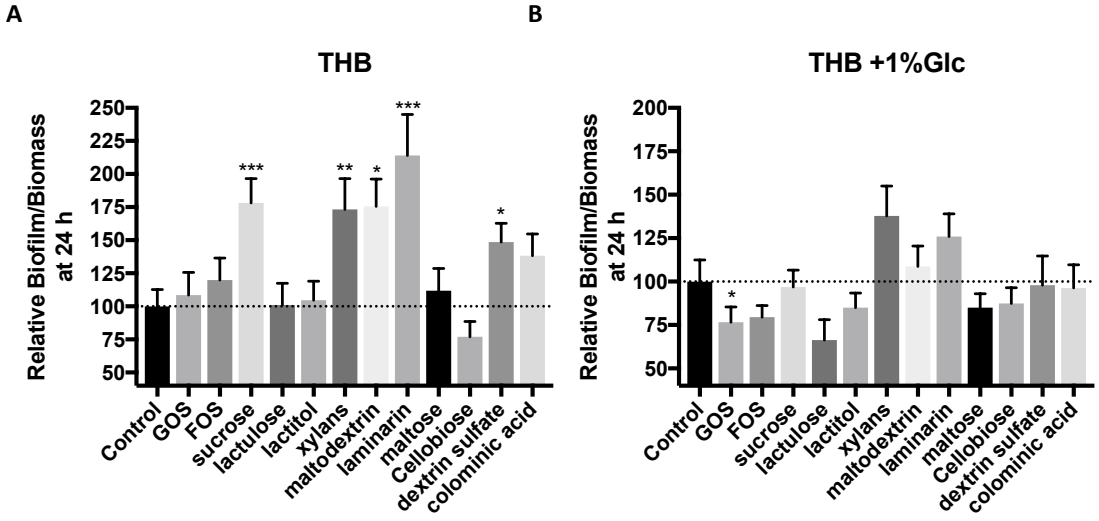


Figure 4. 26 Biofilm/biomass ratios for GB590 at 24 h. was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 13.07 and (B) 7.462 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

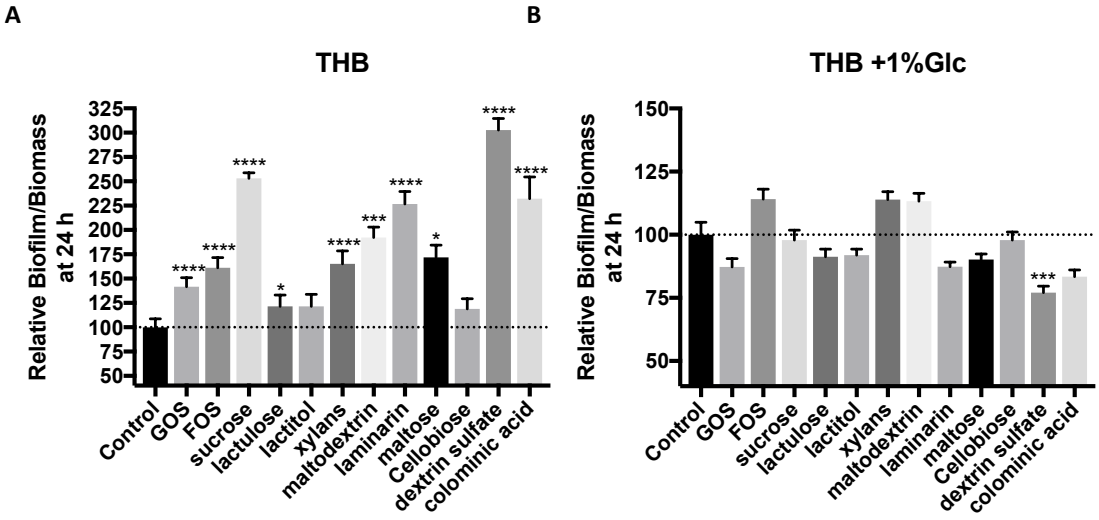


Figure 4. 27 Biofilm/biomass ratios for GB2 at 24 h was compared for (A) THB media or (B) THB media supplemented with 1% glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, F = (A) 45.33 and (B) 17.49 with posthoc Dunnett's multiple comparison test comparing each sample against the control.

Conclusions

We synthesized a number of human milk trisaccharides and unnatural congeners in order to probe the activity of single entity compounds. Our synthetic strategy focused on functionalizing a lactose core at the C3' position using a common lactose acceptor. We developed a robust synthesis of a C3' lactose acceptor that was acetylated at C4' and otherwise perbenzylated. Additionally, each glycosylation reaction was optimized on a gram scale to yield hundreds of milligrams of deprotected trisaccharides to be used for assays testing their biological activity. In doing this, we found that even though 3',4'-lactose diols can exhibit selective nucleophilicity at the equatorial C3' hydroxyl, yields can be improved when there is no competition with the axial C4' hydroxyl.

Our goal was to identify the minimum pharmacophore needed to observe antimicrobial and anti-biofilm activity. We established that lactose and monosaccharide building blocks have no antibacterial activity and little to no anti-biofilm activity against GBS. However, activity begins to surface once an additional monomeric unit is added to the scaffold.

The structure-activity relationship has revealed that both fucose and galactose residues are important for antibacterial activity. Fucosylated HMOs are of particular interest as they account for up to 80% of the oligosaccharides in human milk.[55] Thus, it is notable that the fucosylated trisaccharides exhibit both antimicrobial and anti-biofilm activity in all strains in at least one growth condition. Galactose is also of importance as it is one of the three building blocks that forms the backbone of every HMO. However, GOS, a polymer of galactose residues with glucose at the reducing end, does not exhibit antimicrobial or anti-biofilm activity except under one growth condition for one strain (24% biofilm reduction in GB590 in THB supplemented with 1% glucose).

It would be interesting to explore the effects of a fucose polymer, such as sulfated fucans, on the growth and biofilm of GBS to determine if the activity lies in the lactose core decorated with fucose or in an oligosaccharide containing a fucose residue or branch. Sulfated fucans contain negatively charged sulfate groups which could also influence their antimicrobial or anti-biofilm properties. In the same vein, it is interesting to note that the sialic acid trisaccharide derivatives had no effect on the growth of GBS, but colominic acid, a sialic acid polymer, was able to inhibit growth. This could be due to the ability of colominic acid to better mimic the sialic acid containing capsule of GBS, resulting in a feedback inhibition that inhibits bacterial growth.

Many of the monosaccharides and commercially available oligosaccharides (sucrose, lactulose, lactitol, maltose, and cellobiose) increased the growth of GBS across all strains under at least one growth condition. This indicates that GBS has the ability to utilize smaller carbohydrates for growth and metabolic purposes. Genetic sequencing could reveal if GBS has enzymes specific for these types of saccharides or if the enzymes can act promiscuously to accommodate a wider range of carbon sources.

In terms of anti-biofilm activity, it was of particular interest that the PEGylated derivative showcased notable inhibition of biofilm. Given the length of the PEG linker, this finding supports our previously established hypothesis that larger oligosaccharides are responsible for that mode of activity. Installing PEG branches of varying lengths as well as other poly-ether compounds would provide an interesting route to explore this hypothesis.

While the results obtained in this study were modest in comparison to our previous studies, it is important to note that human milk itself contains a highly variable composition of HMOs. Thus, it is likely that these molecules work in synergy to provide the effects we have previously

observed. Further studies with combinations of individual HMOs could be conducted to probe the synergistic effects that result in increased antimicrobial and anti-biofilm activity.

Experimental Methods

Bacterial Strains and Culture Conditions

All *S. agalactiae* strains (CNCTC 10/84, GB590, GB2) was cultured on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37 °C in ambient air overnight. Bacteria were subcultured from blood agar plates into Todd-Hewitt broth (THB) and incubated under shaking conditions at 37 °C in ambient air overnight. The following day, bacterial density was measured spectrophotometrically using optical density measurements at 600 nm (OD_{600}), and bacterial numbers were determined using the predetermined coefficient of 1 $OD_{600} = 10^9$ CFU/mL.

Bacterial Growth and Biofilm Assays

S. agalactiae strains were grown overnight as described above prior to subculturing 10^6 bacterial cells into 200 µL of THB or THB supplemented with 1% glucose in 96-well tissue culture plates (Corning, Inc.). Bacterial cells were added to wells containing media alone or wells supplemented with 5 mg/mL in individual carbohydrates. Cultures were incubated under static conditions at 37 ° C in ambient air for 24 h. Optical density (OD_{600}) was measured for each sample as a quantification of bacterial growth. The medium was aspirated, and each well was washed once with phosphate-buffered saline (PBS, pH 7.4) to remove nonadherent cells. Wells were then stained with a 10% crystal violet solution for 15 min. After staining, wells were washed with PBS and then allowed to dry at room temperature for 30 min. After drying, crystal violet staining was solubilized with a 4:1 ethanol/acetone solution. The absorbance (OD_{560}) was measured for each sample as a measure of biofilm formation. The data shown represents 3 independent experiments,

each with 3 technical replicates. Results were analyzed compared to controls in the absence of HMOs and expressed a percentage of biomass or biofilm/biomass of the control. Tables show percent change in biofilm/biomass ratio with negative numbers indicating a net decrease in biofilm production and positive numbers indicating a net increase in biofilm production.

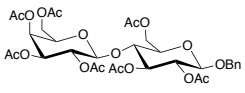
Trisaccharide Synthesis

General Procedure. All non-aqueous reactions were carried out under an inert argon atmosphere with dry solvents under anhydrous conditions, unless otherwise stated. All air- or moisture-sensitive liquids were transferred via disposable or oven-dried stainless syringes. Reaction temperature were monitored and controlled via thermocouple thermometer and corresponding hot plate stirrer. Reactions were conducted at room temperature (approximately 23 °C) unless otherwise noted. Flash column chromatography was performed as described by Still et. al. using silica gel (230-400 mesh). Analytical thin-layer chromatography (TLC) was performed on Sorbtech Silica XHL UV254, glass backed, 250 μm plates or Silicycle SiliaPlate aluminum backed, F-254, 200 μm plates and visualized using cerium ammonium molybdate stain and heat or p-anisaldehyde stain and heat. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous material, unless otherwise stated.

Materials. Dry acetonitrile (MeCN), dichloromethane (DCM), diethyl ether (Et_2O), tetrahydrofuran (THF) and toluene (PhMe) were obtained by passing the previously degassed solvent through activated alumina columns and stored over 4Å or 3Å molecular sieves. Dry triethylamine (Et_3N) was obtained by distillation from CaH_2, followed by storage over KOH pellets. N-iodosuccinimide and N-bromosuccinimide were recrystallized before use. All other reagents were purchased at the highest commercial quality and used without further purification, unless otherwise stated.

Instrumentation: Infrared spectra were obtained as thing films on NaCl plates using a Thermo Scientific Nicolet FT-IR 100 series instrument and are reported in terms of frequency of absorbance (cm^{-1}). ^{1}H NMR spectra were obtained on a Bruker 400 or 600 MHz spectrometers and are reported as follows: chemical shifts (δ ppm), multiplicity (s = singlet, br = broad singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, ddd = doublet of doublet of doublets, m = multiplet), coupling constants (Hz), integration, and assignment. Deuterated chloroform was calibrated to 7.26 ppm. Deuterated methanol was calibrated to 3.31 ppm. Deuterated water was calibrated to 4.79 ppm. ^{13}C NMR spectra were obtained on a Bruker 100 or 150 MHz spectrometer with reporting relative to deuterated solvent signals. Deuterated chloroform was calibrated to 77.16 ppm. Deuterated methanol was calibrated to 49.0 ppm. Assignments were based on homonuclear correlation measurements and DEPT measurements. Low-resolution mass spectra (LCMS) were performed on a Surveyor MSQ spectrometer (Thermo Scientific). High-resolution mass spectra (HRMS) were recorded on a SYNAPT G2 or SYNAPT G2-S spectrometer (Waters) by electrospray ionization time-of-flight (ESI-TOF) reflectron experiments. Optical rotations were obtained using a JASCO P-2000 polarimeter.

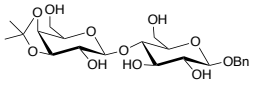
Compound Preparation



9

(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(((2R,3R,4S,5R,6R)-4,5-diacetoxy-2-(acetoxymethyl)-6-(benzyloxy)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyltriacetate (9). [32, 33] To a solution of lactose octaacetate (**8** α,β) (1.0 eq., 6.6 g, 9.7 mmol, α:β 17:83) and benzyl alcohol (1.8 eq., 2.3 mL, 22 mmol) in CH_2Cl_2 (35 mL) at 0 °C was added

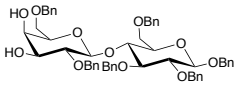
$BF_3 \cdot Et_2O$ (3.7 eq., 3.7 mL, 29 mmol). The reaction warmed to ambient temperature and stirred for 14 h. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with CH_2Cl_2 (2 x 20 mL). The combined organic extracts were washed with sat. aq. $NaHCO_3$ (2 x 10 mL), water (1 x 10 mL), and brine (1 x 10 mL). The organics were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→2:1 hexanes/EtOAc) to provide β-*O*-benzyl peracetylated lactose **9** (3.9 g, 66% relative to β anomer) as a white foam: mp: 66-69 °C; R_f 0.18 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 2964, 1752; ^1H NMR (400 MHz, $CDCl_3$): δ 7.37-7.28 (m, 5H, Ar), 5.35 (dd, J=0.68, 3.28 Hz, 1H, H-4'), 5.16 (dd, J=9.20, 9.32 Hz, 1H, H-3), 5.10 (dd, J=7.88, 10.40 Hz, 1H, H-2'), 4.97 (dd, J=7.84, 9.44 Hz, 1H, H-2), 4.96 (dd, J=3.37, 10.47 Hz, 1H, H-3'), 4.87 (d, J=12.28 Hz, 1H, PhC*H*), 4.60 (d, J=12.28 Hz, 1H, PhC*H*), 4.54-4.50 (m, 1H, H-6b), 4.52 (d, J=7.88 Hz, 1H, H-1'), 4.49 (d, J=7.88 Hz, 1H, H-1), 4.15-4.05 (m, 3H, H-6a', H-6b', H-6a), 3.87 (dt, J=1.04, 6.36 Hz, 1H, H-5'), 3.28 (dd, J=9.28, 9.64, 1H, H-4), 3.58 (ddd, J=2.08, 4.96, 9.92 Hz, 1H, H-5), 2.15 (s, 3H, OAc), 2.14 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.97 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.3, 170.1, 170.0, 169.7, 169.5, 169.0, 136.6, 128.4, 128.0, 127.7, 111.0 (C-1), 99.0 (C-1'), 76.2 (C-4), 72.7 (C-3), 72.6 (C-5), 71.6, 70.9, 70.7 (C-5'), 70.6 (Ph*C*H), 69.0 (C-2'), 66.5 (C-4'), 61.9 (C-6), 60.7 (C-6'), 20.8, 20.7, 20.6, 20.6, 20.4; LRMS calc. for $C_{33}H_{42}O_{18}Na$ $[M+Na]^+$: 749.2, found 749.4.



10

(2R,3R,4R,5S,6R)-2-(benzyloxy)-5-(((3aS,4R,6S,7R,7aR)-7-hydroxy-4-(hydroxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-6-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4-diol (10). [33, 34] To a solution of **9** (1.0 eq., 4.0 g, 5.5 mmol) in MeOH (ca. 300 mL)

was added NaOMe (3.5 mL, 5.4 M). The reaction was stirred 2 h and Dowex 50Wx8 was added until the solution reached a neutral pH. The resulting suspension was filtered and concentrated *in vacuo* to yield a white solid. To a suspension of this white solid (2.4 g, crude) in acetone (ca. 200 mL) was added 2,2-dimethoxy propane (12 eq., 8.0 mL, 65 mmol) and p-toluenesulfonic acid monohydrate (0.1 eq., 0.1 g, 06 mmol). The reaction mixture was stirred 60 h, at which point the solution became homogenous, and was concentrated *in vacuo* and recrystallized from hot ethanol (200 proof) to yield 3',4'-acetonide **10** (1.2 g, 47%) as a white solid: mp: 161-164 °C; R_f 0.43 (9:1 EtOAc/MeOH); IR (thin film, cm^{-1}): 3391, 2923, 2914, 2872, 2866, 2360, 2329; ^1H NMR (400 MHz, MeOD): δ 7.25-7.42 (m, 5H, Ar), 4.92 (d, J=11.80, 1H, PhC*H*), 4.67 (d, J=11.85, 1H, PhC*H*), 4.39 (d, J=7.90, 1H, H-1), 4.37 (d, J=8.28, 1H, H-1'), 4.19 (dd, J=2.04, 5.48, 1H, H-4'), 4.05 (dd, J=5.73, 7.26), 3.92-3.95 (m, 1H, H-5'), 3.92 (dd, J=2.29, 12.13, 1H, H-6a), 3.84 (dd, J=4.20, 12.10, 1H, H-6b), 3.75-3.79 (m, 2H, H-6'), 3.59 (dd, J=8.60, 9.30, 1H, H-4), 3.52 (dd, J=8.60, 9.04, 1H, H-3), 3.45 (dd, J=7.87, 7.73, 1H, H-2'), 3.40 (ddd, J=2.48, 4.12, 9.36, 1H, H-5), 3.32 (m, 1H, H-2), 1.47 (s, 3H, CH_3), 1.32 (s, 3H, CH_3); ^{13}C NMR (100 MHz, MeOD): δ 139.0, 129.3, 129.2, 128.7, 111.1 ($C(CH_3)_2$), 104.2 (C-1), 103.1 (C-1'), 81.0 (C-3), 80.9 (C-3'), 76.5 (C-4), 76.4 (C-5), 75.3 (C-5'), 75.1 (C-4'), 74.9 (C-2), 74.5 (C-2'), 71.8 (Ph*C*H), 62.4 (C-6'), 61.9 (C-6), 28.4 (CH_3), 26.5 (CH_3); LRMS calc. for $C_{22}H_{32}O_{11}Na$ $[M+Na]^+$: 495.2, found 495.3.

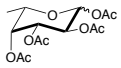


11

(2R,3R,4S,5R,6S)-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-3,4-diol (11). [33, 34] To a solution of **10** (1.0 eq., 1.2 g, 2.6 mmol) in DMF (13 mL) at 0 °C was added benzyl bromide (9.1 eq., 2.8 mL, 24 mmol) followed by NaH (60% wt, 8.3 eq., 0.86 g, 22

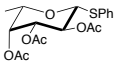
mmol). The reaction stirred for 4 h and was poured into ice and extracted with EtOAc (3 x 50 mL). The combined organic extracts were washed with water (3 x 20 mL) and brine (1 x 20 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to a yellow oil. The crude material was purified by flash column chromatography (hexanes→4:1 hexanes/EtOAc) to yield the perbenzylated acetonide as a clear oil (2.3 g, 96%). A solution of the perbenzylated acetonide (2.3 g, 2.492 mmol) in 80% aq. acetic acid (100 mL) stirred at 65 °C for 4 h. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with CH_2Cl_2 (3 x 50 mL). The combined organic extracts were washed with sat. aq. $NaHCO_3$ (2 x 20 mL) and brine (1 x 20 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude material was purified by flash column chromatography (4:1 hexanes/EtOAc) to yield lactose acceptor **11** (2.0 g, 91%) as a white solid: mp: 109-112 °C; R_f 0.27 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3464, 3063, 3030, 2870; ^1H NMR (400 MHz, $CDCl_3$): δ 7.21-7.38 (m, 30H, Ar), 4.98 (d, J=10.84, 1H, PhC*H*), 4.95 (d, J=11.76, 1H, PhC*H*), 4.91 (d, J=10.84, 1H, PhC*H*), 4.81 (d, 11.60, 1H, PhC*H*), 4.77 (d, 11.08, 1H, PhC*H*), 4.73 (d, J=10.80, 1H, PhC*H*), 4.67 (d, J=11.60, 1H, PhC*H*), 4.66 (d, J=12.08, 1H, PhC*H*), 4.62 (d, J=12.12, 1H, PhC*H*), 4.50 (d, J=7.68, 1H, H-1), 4.47 (d, J=12.08, 1H, PhC*H*), 4.45 (d, J=11.76, 1H, PhC*H*), 4.44 (d, J=7.08, 1H, H-1'), 4.39 (d, J=12.00, 1H, PhC*H*), 4.02 (dd, J=9.16, 9.40, 1H, H-4), 3.95 (t, J=2.76, 1H, H-4'), 3.83 (dd, J=4.08, 10.92, 1H, H-6), 3.57-3.64 (m, 2H, H-3, H-6), 3.48-3.52 (m, 2H, H-2, H-6'), 3.38-3.45 (m, 3H, H-5, H-2', H-3'), 3.36 (q, J=5.72, 1H, H-5'), 2.46 (d, J=3.6, 1H, OH), 2.37 (d, J=4.64, 1H, OH)**;** ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.3, 138.7, 138.5, 138.4, 138.13, 137.7, 128.7, 128.6, 128.5, 128.5, 128.41, 138.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 127.4, 102.72 (C-1'), 102.7 (C-1), 83.0 (C-3), 82.0 (C-2), 80.2 (C-2'), 76.7 (C-4), 75.4 (Ph*C*H), 75.3 (C-5), 75.1 (Ph*C*H), 75.0 (Ph*C*H), 73.7 (C-3'), 73.6 (Ph*C*H), 73.4 (Ph*C*H), 73.0 (C-5'), 71.1 (Ph*C*H), 68.9 (C-4'), 68.8 (C-6'), 68.4 (C-6)**;** LRMS calc. for $C_{54}H_{58}O_{11}Na$ $[M+Na]^+$:

905.4, found 905.5.



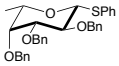
13

(2R,3S,4R,5R,6S)-6-methyltetrahydro-2H-pyran-2,3,4,5-tetrayltetraacetate (13). [36] To a solution of L-fucose (0.71 g, 4.3 mmol) in CH_2Cl_2 (43 mL) was added pyridine (5.1 eq., 1.7 mL, 22 mmol), acetic anhydride (4.9 eq., 2.0 mL, 21 mmol), and 4-dimethylaminopyridine (0.12 eq., 0.060 g, 0.51 mmol). The reaction mixture stirred at ambient temperature for 4 h. The reaction was quenched with 1 M HCl and extracted with CH_2Cl_2 (3 x 20 mL). The combined organic extracts were washed with 1 M HCl (1 x 15 mL), water (1 x 15 mL), and brine (1 x 15 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to yield peracetyaled fucose **12** (1.2 g, 85%, 3:1 α:β ratio) as a clear oil: R_f 0.29 (2:1 hexanes:EtOAc); IR (thin film, cm^{-1}): 2988, 1750, 1224; ^1H NMR (400 MHz, $CDCl_3$) (α anomer): δ 6.33 (d, J=2.80 Hz, 1H, H-1), 5.33-5.37 (m, 3H, H-3, H-4, H-2), 4.27 (q, J=6.48 Hz, 1H, H-5), 2.18 (s, 3H, OAc), 2.15 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.16 (d, J=6.52 Hz, 3H, H-6); (β anomer): δ 5.69 (d, J=8.28 Hz, 1H, H-1), 5.30-5.34 (m, 1H, H-2), 5.27 (dd, J=0.84, 3.40 Hz, 1H, H-4), 5.07 (dd, J=3.44, 10.40 Hz, 1H, H-3), 3.95 (dq, J=0.96, 6.44 Hz, 1H, H-5), 2.18 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.22 (d, J=6.44 Hz, 3H, H-6); ^{13}C NMR (100 MHz, $CDCl_3$) (α anomer): δ 170.7, 170.3, 170.1, 169.3, 90.11 (C-1), 70.7 (C-3), 68.0 (C-4), 67.4 (C-5), 66.6 (C-2), 21.1 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc), 16.1 (C-6); (β anomer): δ 170.7, 170.3, 170.1, 169.3, 92.3 (C-1), 71.4 (C-3), 70.4 (C-5), 70.1 (C-4), 68.1 (C-2), 21.0 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc), 19.1 (C-6); LRMS calc. for $C_{14}H_{20}O_9Na$ $[M+Na]^+$: 355.1, found 354.9.



14

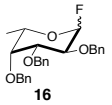
(2S,3R,4R,5S,6R)-2-methyl-6-(phenylthio)tetrahydro-2H-pyran-3,4,5-triyltri acetate (14). [36] To a solution of **13** (1.0 eq., 1.2 g, 3.7 mmol) in CH_2Cl_2 (36 mL) at 0 °C was added thiophenol (2.0 eq., 0.75 mL, 7.4 mmol) and $SnCl_4$ (2.0 eq., 0.86 mL, 7.4 mmol). The reaction warmed to ambient temperature and stirred for 20 h. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with CH_2Cl_2 (2 x 30 mL). The combined organic extracts were washed with sat. aq. $NaHCO_3$ (1 x 15 mL), water (1 x 15 mL), and brine (1 x 15 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude oil was purified by flash column chromatography (toluene→9:1 toluene/EtOAc) to yield **14** (0.87 g, 62%, 1:15 α:β ratio) as a yellow oil: R_f (β anomer) 0.32 (2:1 hexanes/EtOAc), (α anomer) 0.40 (2:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 1749, 1222; ^1H NMR (400 MHz, $CDCl_3$): δ 7.51-7.53 (m, 2H, Ar), 7.31-7.33 (m, 3H, Ar), 5.27 (dd, 1H, J=0.68, 3.32 Hz, H-4), 5.23 (t, 1H, J=9.96 Hz, H-2), 5.06 (dd, 1H, J=3.36, 9.92 Hz, H-3), 4.71 (d, 1H, J=9.92, H-1), 3.84 (dq, 1H, J=0.76, 6.44, H-5), 2.15 (s, 3H, OAc), 2.09 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.25 (d, 3H, J=6.44 Hz, H-6); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.6, 170.1, 169.4, 132.3 (Ph), 128.8 (Ph), 127.9 (Ph), 86.5 (C-1), 73.1 (C-4), 72.4 (C-2), 70.3 (C-3), 67.3 (C-5), 20.8 (OAc), 20.6 (OAc), 20.6 (OAc), 16.4 (C-6); LRMS calc. for $C_{19}H_{27}O_8S$ $[M+CH_3OH+H]^+$: 415.1, found 414.0.



15

(2S,3R,4R,5S,6R)-3,4,5-tris(benzyloxy)-2-methyl-6-(phenylthio)tetrahydro-2H-pyran (15). [36] To a solution of **14** (5.5 g, 14 mmol) in methanol (ca. 90 mL) was added NaOMe (3.0 mL). The reaction stirred for 3 h and Dowex 50Wx8 was added until the solution reached a neutral pH. The resulting suspension was filtered and concentrated *in vacuo*. To a solution of the crude intermediate

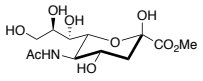
in DMF (50 mL) cooled to 0°C was added benzyl bromide (5.0 eq., 8.5 mL, 72 mmol) followed by Bu_4NI (0.1 eq., 0.62 g, 1.4 mmol). After 10 min of stirring, NaH (60 wt%, 5.0 eq., 2.7 g, 72 mmol) was added in one portion. The reaction was warmed to room temperature and stirred for 16 h. The reaction was poured onto ice and extracted with CH_2Cl_2 (3 x 50 mL). The combined organic extracts were washed with water (1 x 25 mL) and brine (1 x 25 mL), dried ($MgSO_4$), filtered, concentrated *in vacuo*. The resulting crude oil was purified by flash column chromatography (hexanes→4:1 hexanes/EtOAc) to yield **15** (5.7 g, 80%) as a white solid: mp: 103-105 °C; R_f 0.57 (2:1 hexanes/EtOAc), IR (thin film, cm^{-1}): 3062, 3030, 2867, 1124, 1090, 1067; ^1H NMR (400 MHz, $CDCl_3$): δ 7.58-7.61 (m, 2H, Ph), 7.29-7.41 (m, 15, Bn), 7.20-7.22 (m, 3H, Ph), 5.02 (d, 1H, J=11.64 Hz, Bn), 4.80 (d, 1H, J=11.64 Hz, Bn), 4.72-4.75 (m, 3H, Bn), 4.68 (d, 1H, J=11.64, Bn) 4.61 (d, 1H, J=9.68 Hz, H-1) 3.94 (t, 1H, J=9.4 Hz, H-2), 3.65 (d, 1H, J=2.36 Hz, H-4), 3.61 (dd, 1H, J=3.16, 9.16 Hz, H-3), 3.54 (q, 1H, J=6.44 Hz, H-5), 1.28 (d, 3H, J=6.4 Hz, H-6); ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.7, 138.3, 138.3, 134.3, 131.5, 128.7, 128.4, 128.3, 128.5, 128.1, 127.9, 127.6, 127.6, 127.5, 127.4, 126.9, 87.5, 84.5, 77.1, 77.1, 75.5, 74.6, 74.5, 72.8, 17.24 (C-6); LRMS calc. for $C_{33}H_{34}O_4SNa$ $[M+Na]^+$: 549.2, found 549.5.



16

(2S,3S,4R,5R,6S)-3,4,5-tris(benzyloxy)-2-fluoro-6-methyltetrahydro-2H-pyran (16).[36] To a solution of **15** (1.0 eq, 0.1017 g, 0.19 mmol) in CH_2Cl_2 (2.7 mL, 0.07 M) at -50 °C was added NBS (2.7 eq, 0.0915 g, 0.51 mmol) in one portion followed by dropwise addition of DAST (2.0 eq, 0.05 mL, 0.38 mmol). The reaction was monitored by TLC and allowed to stir for 30 m and warm from -45 °C to -10 °C. The reaction was then cooled to -60 °C and quenched with MeOH (1.5 mL) and concentrated *in vacuo*. The resulting oil was dissolved in EtOAc and washed with sat. aq.

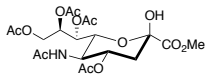
NaS_2O_3 (2 x 10 mL), H_2O (2 x 10 mL), brine (1 x 10 mL), dried over $MgSO_4$, concentrated *in vacuo*, and purified by flash column chromatography (5:1 hexanes/EtOAc) to yield lactose acceptor to yield α-fucosyl fluoride **16** (0.0611 g, 73%) and β-fucosyl fluoride (0.0170 g, 20%) as clear oils: R_f (α) 0.56 (4:1 hexanes/EtOAc), IR (thin film, cm^{-1}): 3032, 2932, 1713, 1240; ^1H NMR (400 MHz, $CDCl_3$): δ 7.42-7.30 (m, 15H, Ph), 5.59 (dd, 1H, J=2.72, 54.1 Hz, H-1), 5.01 (d, 1H, J=11.4 Hz, PhC*H*), 4.87 (dd, 2H, J=2.4, 11.5 Hz, PhC*H*), 4.77 (dd, 2H, J=11.4, 11.5 Hz, PhC*H*), 4.67 (d, 1H, J=11.5 Hz, PhC*H*), 4.11-4.04 (m, 2H, H-2, H-5), 3.96 (dd, 1H, J=2.64, 9.96 Hz, H-3), 3.72 (d, 1H, J=1.68 Hz, H-4), 1.18 (d, 3H, J=6.48 Hz, H-6); ^{13}C NMR (100 MHz, $CDCl_3$): δ 143.9, 138.4, 138.2, 138.0, 132.2, 129.0, 128.3, 127.8, 127.7, 127.6, 125.4, 106.3 (d, J=223 Hz, C-1), 78.7 (C-3), 77.2 (C-4), 75.6 (d, J=23.8 Hz, C-2), 74.9 (Ph*C*H), 73.6 (Ph*C*H), 73.2 (Ph*C*H), 69.1 (d, J=3.17 Hz, C-5), 16.5 (C-6). LRMS calc. for $C_{30}H_{36}NO_5Na$ $[M+ACN+MeOH+Na]^+$: 532.2, found 532.6.



18

methyl(4S,5R,6R)-5-acetamido-2,4-dihydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl) tetrahydro-2H-pyran-2-carboxylate (18).[37] To a suspension of N-acetylneuraminic acid (1.0 eq, 3.3841 g, 10.94 mmol) in methanol (100 mL) was added Dowex50WX8 resin and the resulting slurry stirred at room temperature for 16 h. The resin was removed by filtration and the resulting liquid concentrated *in vacuo* to yield **18** (3.1838 g, 90%) as a white solid: mp 165-167 °C; R_f: 0.43 (4:1 EtOAc/MeOH), IR (thin film, cm^{-1}): 3398, 2360, 2340, 1748, 1635; ^1H NMR (400 MHz, MeOD): δ 8.14 (d, 1H, J=8.56 Hz, N-H), 4.05 (td, 1H, J=4.88, 9.76 Hz, H-4), 4.00 (dd, 1H, J=1.24, 10.52 Hz, H-6), 3.84-3.77 (m, 2H, H-9, H-5), 3.78 (s, 3H, OCH_3), 3.70 (ddd, 1H, J=2.80, 5.56, 8.88 Hz, H-8), 3.62 (dd, 1H, J=5.64, 11.2 Hz, H-9), 3.48 (dd, 1H, J=1.12, 9.08 Hz, H-7), 2.22 (dd,

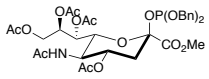
1H, J=4.92, 12.9 Hz, H-3$_{eq}$), 2.02 (s, 3H, OAc), 1.89 (dd, 1H, J=11.4, 12.7 Hz, H-3$_{ax}$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.1 (C-1), 171.8 (Ac), 96.7 (C-2), 72.1 (C-6), 71.7 (C-8), 70.2 (C-7), 67.9 (C-4), 64.9 (C-9), 54.3 (C-5), 53.12(OCH_3), 40.7 (C-3), 22.6 (Ac); LRMS calc. for $C_{12}H_{22}NO_9$ $[M+H]^+$: 324.1, found 324.3.



19

(1S,2R)-1-((2R,3R,4S)-3-acetamido-4-acetoxy-6-hydroxy-6-(methoxycarbonyl)tetrahydro-2H-pyran-2-yl)propane-1,2,3-triyl triacetate (19).[37] To a solution of **18** (1.0 eq, 3.9720 g, 10.5 mmol) in acetyl chloride (42 mL) at 0° C was added methanol (1.0 eq, 0.42 mL, 10.0 mmol) dropwise in a sealed reaction vessel, not allowing any gases formed to escape during the duration of the reaction. The reaction mixture was stirred at room temperature for 17 h. The mixture was diluted with ethyl acetate (50 mL) and washed with sat. $NaHCO_3$ (3 x 100 mL), water (50 mL), and brine (50 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was dissolved in acetone (50 mL) and water (12.5 mL). Ag_2CO_3 (3.1 g) was added in one portion and the slurry was stirred in the dark for 36 h. The solid was filtered, concentrated to ca. 10 mL *in vacuo*, diluted with ethyl acetate and washed with water (50 mL) and brine (50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (1:1 EtOAc/hexanes→2:1 EtOAc/hexanes) to yield **19** (3.5308 g, 68%) as a white solid: mp: 169-170 °C; R_f 0.28 (EtOAc); IR (thin film, cm^{-1}): 3368, 2927, 1714, 1368, 1030; ^{1}H NMR (400 MHz, $CDCl_3$): 5.62 (d, 1H, J=9.48 Hz, N-H), 5.34 (dd, 1H, J=1.44, 5.68 Hz, H-7) 5.25 (ddd, 1H, J=2.44, 5.31, 7.64 Hz, H-8), 5.23-5.18 (m, 1H, H-4), 4.50-4.46 (m, 2H, OH, H-9), 4.18-4.12 (m, 2H, H-6, H-5), 4.02 (dd, 1H, J=7.44, 12.3 Hz, H-9), 3.86 (s, 3H, OCH3), 2.26 (dt, 1H, J=1.68, 12.8 Hz, H-3eq), 2.18 (dd, 1H, J=5.32, 12.8 Hz, H-3ax), 2.14 (s, 3H,

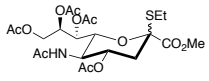
Ac), 2.10 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.90 (s, 3H, Ac); 13C NMR (100 MHz, CDCl3): δ 171.1, 171.1, 171.0, 170.4, 170.3, 169.2, 95.0 (C-2), 71.3 (C-8), 71.2 (C-6), 69.4 (C-4), 68.1 (C-7), 62.8 (C-9), 53.7 (OCH_3), 49.7 (C-5), 36.2 (C-3), 23.3, 21.2, 21.0, 20.9. LRMS calc. for $C_{20}H_{30}NO_{13}$ $[M+H]^+$: 492.2, found 492.3.



20

(1S,2R)-1-((2R,3R,4S)-3-acetamido-4-acetoxy-6-((bis(benzyloxy)phosphanyl)oxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-2-yl)pro pane-1,2,3-triyltriacetate (20).[37] To a solution of **19** (1.0 eq., 86.1 mg, 0.102 mmol) in CH_2Cl_2 (2.0 mL) was added 1*H*-tetrazole (3% wt in CH_3CN, 2.07 mL) and $(BnO)_2PN$*i*Pr_2 (5.5 eq., 0.19 mL, 0.565 mmol) to yield a cloudy solution. The reaction was quenched with 1 M HCl (0.5 mL), and the aqueous layer was extracted with CH_2Cl_2 (3 x 5 mL). The combined organic extracts were washed with water (1 x 2 mL) and brine (1 x 2 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography (1:1 hexanes/EtOAc) to yield **20** (122 mg, 94%) as a white foam: mp: 31-33 °C; R_f 0.45 (EtOAc); IR (thin film, cm^{-1}): 3054, 2987, 1748, 1265; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50-7.52 (m, 2H, Ar) 7.42-7.46 (m, 2H, Ar), 7.30-7.38 (m, 6H, Ar), 5.20 (dd, J=9.84, 13.76, 1H, PhC*H*), 5.14-5.16 (m, 2H, H-8, H-7), 4.94-4.50 (m, 2H, PhC*H*), 4.85-4.92 (m, 2H, PhC*H*, H-4), 4.58 (dd, J=2.2, 12.66, 1H, H-9) 4.31 (d, J=10.4, 1H, NH), 4.10-4.15 (m, 1H, H-9), 4.01 (q, J=10.52, 1H, H-5), 3.73 (s, 3H, OCH_3), 2.41 (dd, J=4.96, 13.04, 1H, H-3_{eq}), 2.00-2.12 (m, 1H, H-3_{ax}), 2.09 (s, 6H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.80 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.7, 170.6, 170.5, 170.2, 167.0, 167.8, 138.8 (d, J=2.2), 138.1 (d, J=5.5), 128.9, 128.6, 128.2, 128.0, 127.9, 127.8, 98.0 (d, J=7.3, C-2), 72.4, (C-6), 72.0 (C-8), 68.5 (C-4), 62.8 (C-9), 53.3 (O*C*H_3), 48.5 (C-5), 37.9 (C-3); LRMS calc. for $C_{34}H_{42}NO_{15}PNa$ $[M+Na]^+$: 758.2,

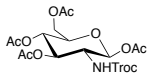
found 758.3.



21

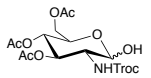
(1S,2R)-1-((2R,3R,4S)-3-acetamido-4-acetoxy-6-(ethylthio)-6-(methoxycarbonyl)tetrahydro-2H-pyran-2-yl)propane-1,2,3-triyl triacetate (21).[39] To a solution of **18** (1.0 eq, 1.3 g, 4.0 mmol) in pyridine (20 mL) was added acetic anhydride (10 eq, 4.2 mL, 44 mmol) then DMAP (0.1 eq, 51.1 mg, 0.418 mmol). The reaction mixture stirred under argon atmosphere at 0 °C and warming to room temperature overnight. The reaction was quenched with $CuSO_4$ and extracted with CH_2Cl_2 (50 mL). The organic extracts were washed with water (3 x 20 mL), then CuSO4 (1 x 20 mL), water (3 x 20 mL), brine (1 x 20 mL), dried over $MgSO_4$, and concentrated to yield a peracetylated intermediate. To the crude intermediate in CH_2Cl_2 (6.5 mL) at 0 °C was added ethane thiol (0.175 mL, 2.4 mmol) then BF_3•Et_2O (0.33 mL, 2.7 mmol). The reaction mixture was allowed to warm to room temperature and stir for 17 h. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with CH_2Cl_2. The organic extracts were washed with $NaHCO_3$ (2 x 10 mL) and brine (1 x 10 mL), dried over $MgSO_4$, and concentrated to a white foam. The foam was purified over flash column chromatography (4:1 hexanes:EtOAc→EtOAc) to yield **21** as a white foam (0.89 g, 42%): mp: 75-77 °C; R_f 0.57 (EtOAc); IR (thin film, cm^{-1}): 2967, 1745, 1663, 1545, 1228; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.51 (d, 1H, J=10.2 Hz, N-H), 5.42 (d, 1H, J=2.49 Hz, H-7), 5.32-5.22 (m, 1H, H-4), 5.11 (dt, 1H, J=2.60, 8.00 Hz, H-8), 4.78 (dd, 1H, J=2.40, 12.4 Hz, H-9), 4.33 (dd, 1H, J=2.36, 7.60 Hz, H-6), 4.14 (dd, 1H, J=8.08, 12.3 Hz, H-9), 4.07 (q, 1H, J=10.5 Hz, H-5), 3.79 (s, 3H, OC*H*$_3$), 2.63-2.46 (m, 3H, H-3$_{eq}$, SC*H*$_2$CH$_3$), 2.15 (d, 1H, J=4.16 Hz, H-3$_{ax}$), 2.11 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.86 (s, 3H, Ac), 1.19 (t, 3H, J=7.52 Hz, SCH$_2$C*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.1, 171.0, 170.6, 170.3, 170.3, 168.5, 85.1, 72.7, 72.3, 69.4,

68.7, 62.6, 53.0, 49.6, 37.4, 23.2, 22.8, 21.1, 21.0, 20.9, 20.8, 14.2.



23

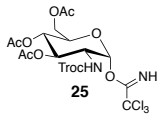
(2*S*,3*R*,4*R*,5*S*,6*R*)-6-(acetoxymethyl)-3-(((2,2,2trichloroethoxy)carbonyl)amino)tetrahydro-2*H*-pyran-2,4,5-triyltriacetate (23).[40] To a solution of D-glucosamine hydrochloride (1.17 g, 3.30 mmol) in pyridine (15 mL) was added acetic anhydride (13 eq., 4 mL, 42.3 mmol) and a catalytic amount of 4-dimethylaminopyridine. The reaction stirred for 17 h and was quenched with 1 M HCl (15 mL) and extracted with CH_2Cl_2 (3 x 20 mL). The combined organic extracts were washed with 1 M HCl (2 x 10 mL), sat. aq. $NaHCO_3$ (1 x 10 mL), water (1 x 10 mL), and brine (1 x mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to yield **23** (1.58 g, 92%) as a white foam: mp: 35-38 °C; R_f 0.21 (2:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3328, 2960, 1753, 1225; ^1H NMR (400 MHz, $CDCl_3$) (α anomer): δ 6.24 (d, J=3.65, 1H, H-1), 5.28 (dd, J=9.76, 10.50, 1H, H-3), 5.20 (dd, J=9.62, 9.89, 1H, H-4), 5.11 (d, J=9.42, H1, NH), 4.82 (d, J=12.13, 1H, Troc-C*H*), 4.62 (d, J=12.05, Troc-C*H*), 4.28 (d, J=4.09, 12.51, 1H, H-6), 4.21 (d, J=3.71, 9.73, 1H, H-2), 4.06 (d, J=2.14, 12.38, 1H, H-6), 4.01 (Troc-CH_2), 2.21 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), (β anomer): δ 5.74 (d, J=8.76, 1H, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.4, 170.8, 169.3, 168.7, 154.2, 95.4 (Troc-*C*Cl_3), 90.6 (C-1), 74.8 (Troc-*C*H_2), 70.5 (C-3), 69.9 (C-5), 67.6 (C-4), 61.6 (C-6), 53.4 (C-2), 21.1 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc); LRMS calc. for $C_{19}H_{25}Cl_3N_2O_{11}Na$ $[M+ACN+Na]^+$: 585.0, found 585.1.



24

(2R,3S,4R,5R)-2-(acetoxymethyl)-6-hydroxy-5-(((2,2,2-trichloroethoxy)carbonyl)amino)

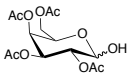
tetrahydro-2H-pyran-3,4-diyldiacetate (24).[40] To a solution of **23** (1.0 eq, 3.2 g, 6.1 mmol) in THF (30 mL, 0.2 M) was added dimethylaminopropylamine (5.0 eq., 3.8 mL g, 30 mmol). The reaction stirred at room temperature for 110 min and was quenched with 1 M HCl (50 mL). The reaction was diluted with CH_2Cl_2, washed with 1 M HCl (2 x 30 mL) and brine (30 mL), dried over $MgSO_4$, filtered, concentrated *in vacuo* to yield **24** (2.8 g, 95%) as a white solid: mp: 175-178 °C; R_f (α anomer) 0.17 (2:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3332, 1747, 1237; ^1H NMR (400 MHz, $CDCl_3$): δ 5.34-5.36 (m, 2H, H-3, H-1), 5.13 (t, J=9.56, 1H, H-5), 4.79 (d, J=12.00, 1H, Troc-C*H*), 4.64 (d, J=12.04, 1H, Troc-C*H*), 4.21-4.29 (m, 2H, H-4, H-6), 4.12-4.17 (m, 1H, H-6), 4.06 (dt, J=3.60, 10.44, 1H, H-2), 3.13 (d, J=2.56, 1H, OH), 2.10 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.01 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.12, 170.94, 169.59, 154.35, 95.47 (Troc-*C*Cl_3), 92.00 (C-1), 74.76 (Troc-*C*H_2), 70.79 (C-3), 68.37 (C-5), 67.99 (C-4), 62.14 (C-6), 54.29 (C-2), 20.93 (OAc), 20.85 (OAc), 20.78 (OAc); LRMS calc. for $C_{15}H_{20}Cl_3NO_{10}Na$ $[M+Na]^+$: 502.01, found 501.15.



25

(2R,3S,4R,5R,6R)-2-(acetoxymethyl)-6-(2,2,2-trichloro-1-iminoethoxy)-5-(((2,2,2-trichloroethoxy)carbonyl)amino)tetrahydro-2H-pyran-3,4-diyl diacetate (25).[40] To a solution of **24** (1.0 eq, 3.2 g, 6.7 mmol) in CH_2Cl_2 (100 mL, 0.07 M) at 0 °C was added trichloroacetonitrile (10 eq., 6.7 mL, 67 mmol) and DBU (0.25 eq, 0.25 mL, 1.7 mmol). The reaction was stirred at room temperature for 3 h. The reaction was concentrated *in vacuo*, and purified by flash column chromatography (4:1 hexanes/EtOAc) to yield **25** (2.9 g, 70%) as a white foam: mp: 58-62 °C; R_f 0.29 (2:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3310, 1749, 1226; ^1H NMR (400 MHz, $CDCl_3$): δ

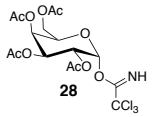
8.80 (s, 1H, N-H), 6.43 (d, J=3.56, 1H, H-1), 5.35 (dd, J=9.76, 10.56, 1H, H-3), 5.25 (dd, J=9.76, 10.04, 1H, H-5), 5.17 (d, J=9.24, 1H, N-H), 4.74 (d, J=12.04, 1H, Troc), 4.70 (d, J=12.04, 1H, Troc), 4.26-4.32 (m, 2H, H-2, H-6), 4.10-4.16 (m, 2H, H-4, H-6), 2.08 (s, 3H, OAc), 2.06 (s, 6H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.3, 170.7, 169.4, 160.6, 154.3, 95.4 (Troc-*C*Cl_3), 94.7 (C-1), 90.6, 74.8 (Troc-*C*H_2), 70.4 (C-3, C-4), 67.5 (C-5), 61.5 (C-6), 54.0 (C-2), 20.8 (OAc), 20.7 (OAc); LRMS calc. for $C_{17}H_{20}Cl_6N_2O_{10}Na$ $[M+Na]^+$: 644.9, found 645.2.



27

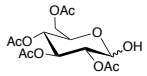
(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-hydroxytetrahydro-2H-pyran-3,4,5-triyl triacetate (27). To a solution of β-D-galactose pentaacetate (1.0 eq, 2.04 g, 5.23 mmol) in THF (26 mL, 0.2 M) was added 3-(dimethylamino)-1-propylamine (5.0 eq, 3.26 mL). The reaction stirred for 90 min. The reaction was quenched with 1 M HCl (50 mL) and diluted with CH_2Cl_2 (80 mL). The organic layer was washed with 1 M HCl (2 x 30 mL) and brine (1 x 30 mL), dried ($MgSO_4$), and concentrated *in vacuo* to yield **27** (1.58 g, 87%, 5:2 β:α ratio) as an opaque gel: R_f: 0.22 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3388, 3040, 2972, 2243, 1739; ^{1}H NMR (400 MHz, $CDCl_3$): β anomer: δ 5.53 (t, J=7.00 Hz, 1H, H-1), 5.48 (dd, J=1.16, 3.32 Hz, 1H, H-4), 5.41 (dd, J=3.28, 10.81 Hz, 1H, H-3), 5.17 (ddd, J=1.04, 3.80, 11.08, 1H, H-2), 4.47 (dt, J=0.80, 6.60 Hz, 1H, H-5), 4.16-4.05 (m, 2H, H-6), 2.91 (dd, J=1.12, 3.48 Hz, 1H, OH), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.06 (s, 3H, OAc), 1.99 (s, 3H, OAc); α anomer: δ 5.41 (m, 1H, H-3), 5.07 (m, 1H, H-2), 4.69 (m, 1H, H-1), 4.16-4.05 (m, 3H, H-5, H-6), 3.96 (dt, J=1.08, 6.56 Hz, 1H, H-4), 3.50 (d, J=9.08 Hz, 1H, OH), 2.16 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.00 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): β anomer: δ 170.7, 170.5, 170.4, 170.2, 90.9 (C-1), 68.4 (C-2), 68.3 (C-4), 67.2 (C-3), 66.5 (C-5), 62.0 (C-6), 21.0 (OAc), 20.9 (OAc), 20.8 (OAc), 20.8 (OAc); α anomer: δ

96.2 (C-1), 71.3 (C-4), 71.1 (C-2), 70.4 (C-5), 67.3 (C-3), 61.6 (C-6); LRMS calc. for $C_{16}H_{23}NO_{10}Na$ $[M+MeCN+Na]^+$: 412.1, found 412.2.



28

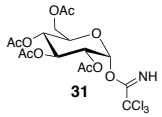
(2R,3S,4S,5R,6R)-2-(acetoxymethyl)-6-(2,2,2-trichloro-1-iminoethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (28). To a solution of **27** (1.0 eq, 1.58 g, 4.53 mmol) in CH_2Cl_2 (45 mL, 0.1 M) was added trichloroacetonitrile (10 eq, 4.60 mL) and DBU (0.25 eq, 0.171 mL). The reaction stirred for 2 h and was concentrated *in vacuo*. The crude residue was purified by flash column chromatography (4:1 hexanes/EtOAc) to yield **28** (1.85 g, 83%) as a white solid: mp: 103-105 °C; R_f: 0.41 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3303, 1743; ^1H NMR (400 MHz, $CDCl_3$): δ 8.66 (s, 1H, N-H), 6.60 (d, J=3.48 Hz, 1H, H-1), 5.56 (dd, J=1.08, 3.08 Hz, 1H, H-4), 5.43 (dd, J=3.12, 10.84 Hz, 1H, H-3), 5.36 (dd, J=3.48, 10.84 Hz, 1H, H-2), 4.44 (t, J=6.52 Hz, 1H, H-5), 4.17 (dd, J=6.64, 11.32 Hz, 1H, H-6), 4.08 (dd, J=6.68, 11.32 Hz, 1H, H-6), 2.17 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc) ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.5, 170.2, 170.2, 170.1, 161.1 (CCl_3), 93.7 (C-1), 90.9 (*C*=NH), 69.1 (C-5), 67.7 (C-3), 67.5 (C-4), 67.1 (C-2), 61.4 (C-6), 20.9 (OAc), 20.8 (OAc), 20.8, (OAc), 20.7 (OAc); LRMS calc. for $C_{18}H_{23}Cl_3N_2O_{10}Na$ $[M+MeCN+Na]^+$: 555.0, found 555.2.



30

(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-hydroxytetrahydro-2H-pyran-3,4,5-triyl triacetate (30). To a solution of β-D-glucose pentaacetate (1.0 eq, 2.17 g, 5.56 mmol) in THF (30 mL, 0.2 M) was added 3-(dimethylamino)-1-propylamine (5.0 eq, 3.46 mL). The reaction stirred for 90

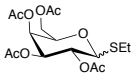
min. The reaction was quenched with 1 M HCl (50 mL) and diluted with CH_2Cl_2 (80 mL). The organic layer was washed with 1 M HCl (2 x 30 mL) and brine (1 x 30 mL), dried ($MgSO_4$), and concentrated *in vacuo* to yield **30** (1.90 g, 98%, 5:2 β:α ratio) as an opaque gel: R_f: 0.23 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3405, 1743, 1641; ^1H NMR (400 MHz, $CDCl_3$): β anomer: δ 5.53 (t, J=7.00 Hz, 1H, H-1), 5.48 (dd, J=1.16, 3.32 Hz, 1H, H-4), 5.41 (dd, J=3.28, 10.81 Hz, 1H, H-3), 5.17 (ddd, J=1.04, 3.80, 11.08 Hz, 1H, H-2), 4.47 (dt, J=0.80, 6.60 Hz, 1H, H-5), 4.16-4.05 (m, 2H, H-6), 2.91 (dd, J=1.12, 3.48 Hz, 1H, OH), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.06 (s, 3H, OAc), 1.99 (s, 3H, OAc); α anomer: δ 5.41 (m, 1H, H-3), 5.07 (m, 1H, H-2), 4.69 (m, 1H, H-1), 4.16-4.05 (m, 3H, H-5, H-6), 3.96 (dt, J=1.08, 6.56 Hz, 1H, H-4), 3.50 (d, J=9.08 Hz, 1H, OH), 2.16 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.00 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): β anomer: δ 170.7, 170.5, 170.4, 170.2, 90.9 (C-1), 68.4 (C-2), 68.3 (C-4), 67.2 (C-3), 66.5 (C-5), 62.0 (C-6), 21.0 (OAc), 20.9 (OAc), 20.8 (OAc), 20.8 (OAc); α anomer: δ 96.22 (C-1), 71.3 (C-4), 71.1 (C-2), 70.4 (C-5), 67.3 (C-3), 61.6 (C-6); LRMS calc. for $C_{14}H_{24}NO_{10}$ $[M+NH_4]^+$: 366.1, found 366.3.



31

(2R,3R,4S,5R,6R)-2-(acetoxymethyl)-6-(2,2,2-trichloro-1-iminoethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (31). To a solution of **30** (1.0 eq, 1.56 g, 4.52 mmol) in CH_2Cl_2 (45 mL, 0.1 M) was added trichloroacetonitrile (10 eq, 4.6 mL, 45 mmol) and DBU (0.25 eq, 0.171 mL, 1.13 mmol). The reaction stirred for 2 h and was concentrated *in vacuo*. The crude residue was purified by flash column chromatography (4:1 hexanes/EtOAc) to yield imidate **30** (1.84 g, 83%) as a white solid: mp: 33-35 °C; R_f: 0.70 (1:1 hexanes/EtOAc) IR (thin film, cm^{-1}): 3340,

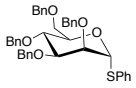
1763, 1677; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.69 (s, 1H, N-H), 6.56 (d, J=3.68 Hz, 1H, H-1), 5.56 (t, J= 9.84 Hz, 1H, H-4), 5.18 (dd, J=9.72, 10.0 Hz, 1H, H-3), 5.13 (dd, J=3.72, 10.2 Hz, 1H, H-2), 4.27 (dd, J=4.12, 12.3 Hz, 1H, H-6), 4.21 (ddd, J=1.88, 4.08, 10.2 Hz, 1H, H-5), 4.13 (dd, J=2.00, 12.3 Hz, 1H, H-6), 2.07 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc) ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.7, 170.1, 167.0, 169.6, 160.9 (*C*Cl_3), 93.0 (C-1), 90.8 (*C*=NH), 70.1 (C-5), 70.0 (C-4, C-2), 67.9 (C-3), 61.5 (C-6), 20.8 (OAc), 20.7 (OAc), 20.6, (OAc); LRMS calc. for $C_{16}H_{24}Cl_3N_2O_{10}Na$ $[M+NH_4]^+$: 509.1, found 508.6.



32

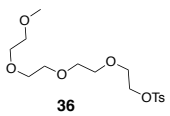
(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(ethylthio)tetrahydro-2H-pyran-3,4,5-triyl triacetate (32).[42] To a solution of **26** (3.01 g, 7.72 mmol, 1 eq.) in CH_2Cl_2 (40 mL, 0.19 M) cooled to 0 °C was added ethanethiol (0.740 mL, 10 mmol, 1.3 eq) then $BF_3 \cdot Et_2O$ (1.50 mL, 11.8 mmol, 1.5 eq.). The reaction warmed naturally and stirred for 17 h. The reaction was quenched with satturated $NaHCO_3$, and the product extracted with CH_2Cl_2 (2 x 10 mL). The organic extracts were combined and washed with sat. aq. $NaHCO_3$ (20 mL), water (20 mL), and brine (20 mL), dried over $MgSO_4$, concentrated, and purified by flash column chromatography (4:1 hexanes/EtOAc) to yield **31** (2.96 g, 98%, 6:5 α:β ratio) as a white solid: R_f: 0.21 (1:2 hexanes/EtOAc); IR (film): 3476, 2971, 2934, 1751, 1371 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.43 (dd, 1H, J=0.88, 3.36 Hz, H-4), 5.24 (t, 1H, J=9.99, H-2), 5.05 (dd, 1H, J=3.36, 10.0 Hz, H-3), 4.49 (d, 1H, J=9.96 Hz, H-1), 4.17 (dd, 1H, J=6.66, 11.3 Hz, H-6), 4.10 (dd, 1H, J=6.60, 11.3 Hz, H-6), 3.93 (dt, 1H, J=0.96, 6.60 Hz, H-5), 2.79-2.65 (m, 2H, C*H*$_2$CH$_3$), 2.15 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.98 (s, 3H, Ac) 1.28 (t, 3H, J=7.44 Hz, CH$_2$C*H*$_3$) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.5, 170.4, 170.2, 169.7, 84.2 (C-1), 74.6 (C-5), 67.4 (C-2), 67.4 (C-4), 61.6 (C-6), 24.5 (*C*H_2CH_3), 21.0 (Ac), 20.8 (Ac),

20.7 (Ac), 15.0 (CH_2CH_3); LRMS calc. for $C_{16}H_{24}O_9SNa$ $[M+Na]^+$: 415.1, found 415.1.



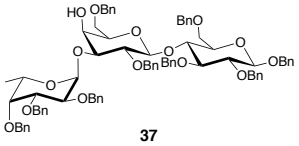
34

(2R,3R,4S,5S,6R)-3,4,5-tris(benzyloxy)-2-((benzyloxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran (34). To a solution of **33** (0.137 g, 0.504 mmol) and benzyl bromide (5.5 eq., 0.330 mL, 2.78 mmol) in DMF (5 mL) cooled to 0 °C was added NaH (60 wt%, 7.1 eq., 0.143 g, 3.59 mmol) in one portion. The reaction mixture was warmed to room temperature and stirred for 3 h. The reaction was quenched with water and extracted with EtOAc (3x). The combined organic extracts were washed with water (2x) and brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and purified by flash column chromatography (hexanes→4:1 hexanes/EtOAc) to yield **34** as a clear oil (0.300 g, 94%): R_f: 0.39 (4:1 hexanes/EtOAc); IR (film): 3062, 3030, 2899 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.19-7.46 (m, 30H, Ar), 5.61 (d, J=1.52, 1H, H-1), 4.91 (d, J=10.76, 1H, PhC*H*), 4.73 (d, J=12.36, 1H, PhC*H*), 4.65 (d, J=11.92 1H, PhC*H*), 4.64 (d, J=12.40, 1H, PhC*H*), 4.63-4.58 (m, 2H, PhC*H*), 4.54 (d, J=10.84, 1H, PhC*H*), 4.49 (d, J=11.96, 1H, PhC*H*), 4.27 (ddd, J=2.04, 5.36, 10.2, 1H, H-5), 4.07 (t, J=9.44, 1H, H-4) 4.00 (dd, J=1.88, 3.00, 1H, H-2), 3.87 (dd, J=3.08, 9.26, 1H, H-3), 3.84 (dd, J=5.50, 10.81, 1H, H-6), 3.75 (dd, J=1.84, 10.88, 1H, H-6); ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.6, 138.3, 138.1, 131.8, 129.1, 128.6, 128.5, 128.5, 128.4, 128.1, 128.1, 128.0, 127.9, 127.8, 127.8, 127.6, 127.5, 85.9 (C-1), 80.3 (C-3), 76.4 (C-2), 75.3 (Ph*C*H), 75.2 (C-4), 73.4 (Ph*C*H), 72.9 (C-5), 72.3 (Ph*C*H), 72.0 (Ph*C*H), 69.4 (C-6); LRMS calc. for $C_{40}H_{44}NO_5S$ $[M+NH_4]^+$: 650.3, found 650.4.



36

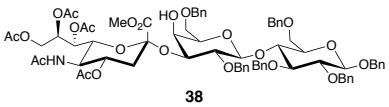
2,5,8,11-tetraoxatridecan-13-yl 4-methylbenzenesulfonate (36). To a solution of tetraethyleneglycol monomethyl ether (0.376 g, 1.81 mmol) in CH_2Cl_2 (4.10 mL) was added TsCl (0.753 g, 3.95 mmol, 2.2 eq) and pyridine (0.220 mL). The reaction stirred at room temperature for 40 hours, was diluted with excess CH_2Cl_2 and quenched with 1 M NaOH, washed with sat'd. $NaHCO_3$, water, and brine, dried over $MgSO_4$, and concentrated to a clear oil. The oil was purified by flash column chromatography (1:1 EtOAc/hexanes to EtOAc) to yield **35** (0.621 g, 95%) as a clear liquid: R_f 0.55 (EtOAc); IR (thin film, cm^{-1}): 3450, 2864, 1590, 1350; ^1H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, 2H, J=8.32 Hz, Ar), 7.33 (d, 2H, J=8.08 Hz, Ar), 4.15 (dd, 2H, J=4.80, 5.80 Hz), 3.67 (dd, 2H, J=3.92, 4.92 Hz), 3.63-3.60 (m, 6H), 3.57 (m, 4H), 3.54-3.52 (m, 2H), 3.36 (s, 3H, OC*H*$_3$), 2.43 (s, 3H, Ar-C*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 144.9, 133.1, 129.9, 128.1, 72.0, 70.8, 70.7, 69.4, 68.8, 59.1 (O*C*H_3), 21.7 (Ar-*C*H_3); LRMS calc. for $C_{16}H_{26}O_7SNa$ $[M+Na]^+$: 385.2, found 385.1.



37

(2R,3S,4S,5R,6S)-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)-4-(((2S,3S,4R,5R,6S)-3,4,5-tris(benzyloxy)-6-methyltetrahydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3-ol (37). A solution **15** (1.2 eq, 74.5 mg, 0.141 mmol) and **11** (1.0 eq, 105 mg, 0.119 mmol) in Et_2O/CH_2Cl_2 (5:1, 1.2 mL) was stirred over freshly activated 4Å MS for 1 h. The solution was cooled to -40 °C and NIS (2.4 eq, 0.0638 g, 0.284 mmol) and AgOTf (1.0 eq, 0.0336 g, 0.131 mmol) were added. The reaction warmed to ambient temperature and stirred for 5 h. The reaction was quenched with triethylamine, filtered through a plug of Celite using additional CH_2Cl_2,

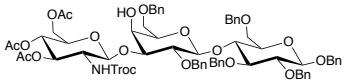
washed with sat. aq. Na_2SO_4 (2 x 10 mL) and brine (1 x 10 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→40% EtOAc/hexanes) to yield **37α** (63.6 mg, 41%) and **37β** (32.0 mg, 21%) as white solids: mp (α): 45-46 °C; R_f (α) 0.51 (2:1 hexanes:EtOAc); IR (thin film, cm^{-1}, α): 3465, 3063, 3030, 2870; ^1H NMR (600 MHz, $CDCl_3$): (α) δ 5.25 (d, J=3.60, 1H, H-1''), 4.96 (d, J=11.52, 1H, PhC*H*), 4.95 (d, J=10.86, 1H, PhC*H*), 4.92 (d, J=12.06, 1H, PhC*H*), 4.88 (d, J=10.86, 1H, PhC*H*), 4.83 (d, J=11.70, 1H, PhC*H*), 4.82 (d, J=11.88, 1H, PhC*H*), 4.73 (d, J=11.76, 1H, PhC*H*), 4.72 (d, J=10.80, 1H, PhC*H*), 4.71 (d, J=10.92, 1H, PhC*H*), 4.70 (d, J=11.88, 1H, PhC*H*), 4.64 (d, J=11.52, 1H, PhC*H*), 4.62 (d, J=12.06, 1H, PhC*H*), 4.57 (d, J=12.18, 1H, PhC*H*), 4.56 (d, J=12.00, 1H, PhC*H*), 4.44 (d, J=12.06, 1H, PhC*H*), 4.43 (d, J=7.62 1H, H-1), 4.41 (d, J=7.38, 1H, H-1'), 4.39 (d, J=11.88, 1H, PhC*H*), 4.34 (d, J=12.12, 1H, PhC*H*), 4.07 (q, J=6.36, 1H, H-5''), 4.04 (dd, J=3.66, 10.2, 1H, H-4''), 3.97-4.00 (m, 2H, H-2'', H-4), 3.92 (d, J=2.64, 1H, H-4'), 3.75 (dd, J=3.96, 10.92, 1H, H-6), 3.70 (d, J=1.32, 1H, H-3''), 3.63 (dd, J=6.96, 9.84, 1H, H-6'), 3.44-3.61 (m, 6H, H-2', H-6, H-3', H-3, H-6', H-2), 3.34 (t, J=6.12, 1H, H-5'), 3.22 (ddd, J=1.68, 3.78, 9.90, 1H, H-5), 2.41 (s, 1H, O-H), 1.15 (d, J=6.48, 3H, H-6''); ^{13}C NMR (150 MHz, $CDCl_3$): δ 139.3, 139.1, 138.9, 138.8, 138.7, 138.6, 138.4, 138.3, 137.7, 128.6, 128.5, 128.5, 128.4, 128.4, 128.3, 128.2, 128.2, 128.0, 128.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.5, 127.4, 127.2, 127.1, 102.7 (C-1'), 102.6, (C-1), 99.4 (C-1''), 83.2 (C-3), 81.9 (C-2), 80.3 (C-3'), 79.3 (C-2''), 79.2 (C-2'), 77.8 (C-3''), 76.5 (C-4), 75.9 (C-4''), 75.5 (Ph*C*H), 75.2 (Ph*C*H), 75.1 (C-5), 75.1 (Ph*C*H), 74.6 (Ph*C*H), 73.5 (Ph*C*H), 73.1 (Ph*C*H), 72.8 (H-5'), 71.1 (Ph*C*H), 69.0 (C-4'), 68.6 (C-6'), 68.1 (C-6), 67.2 (C-5''), 16.9 (C-6''); HRMS calc. For $C_{81}H_{86}O_{15}Na$ $[M+Na]^+$: 1321.5864, found 1321.5773.



38

(1S,2R)-1-((2R,3R,4S,6S)-3-acetamido-4-acetoxy-6-(((2S,3R,4S,5S,6R)-3-(benzyloxy)-6-((benzyloxy)methyl)-5-hydroxy-2-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-2-yl)propane-1,2,3-triyl triacetate (38). A solution of **20** (1.0 eq., 50.8 mg, 0.069 mmol) and **11** (1.3 eq., 79.3 mg, 0.0900 mmol) in proprionitrile (2.0 mL) was stirred over freshly activated 4Å MS for 30 min. The solution was cooled to -30 °C and trimethylsilyl trifluoromethanesulfonate (1 drop) was added. The reaction slowly warmed to ambient temperature and stirred for 24 h. The reaction was quenched with triethylamine, filtered through a plug of Celite using additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (1:1 hexanes/EtOAC→ EtOAc) to yield **38** (42.1 mg, 44.9%) as a white solid: mp: 57-60°C; R_f 0.35 (1:3 hexanes:EtOAc); IR (thin film, cm^{-1}): 3030, 2867, 1747; ^1H NMR (400 MHz, $CDCl_3$): δ 7.19-7.41 (m, 30H, Ar), 5.41 (ddd, J=2.56, 5.84, 8.16, 1H, H-8''), 5.31 (dd, J=2.08, 8.08, 1H, H-7''), 5.08 (d, J-9.92, 1H, N-H), 4.98 (d, J=10.76, 1H, PhC*H*), 4.94 (d, J=12.04, 1H, PhC*H*), 4.90 (d, J=10.92, 1H, PhC*H*), 4.83-4.90 (m, 1H, H-4''), 4.78 (d, J=11.76, 1H, PhC*H*), 4.74 (d, J=10.52, 1H, PhC*H*), 4.72 (d, J=10.88, 1H, PhC*H*), 4.68 (d, J=12.32, 1H, PhC*H*), 4.64 (d, J=12.36, 1H, PhC*H*), 4.58 (d, J=7.56, H-1'), 4.42-4.52 (m, 3H, PhC*H*), *4*.47 (d, J=7.72, 1H, H-1), 4.34 (d, J=11.92, 1H, PhC*H*), 4.29 (dd, J=2.48, 12.40, 1H, H-9''), 3.94-4.13 (m, 5H, H-5'', H-3', H-6'', H-4, H-9''), 3.83 (t, J=3.40, 1H, H-4'), 3.76 (s, 3H, OCH_3), 3.62-3.77 (m, 3H, H-6, H-6'), 3.45-3.58 (m, 5H, H-3, H-2', H-5', H-6', H-2), 3.34-3.38 (m, 1H, H-5), 2.68 (d, J=3.32, 1H, O-H), 2.51 (dd, J=4.72, 13.04, 1H, H-2''), 2.00-2.08 (m, 1H, H-2''), 2.09 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.90 (s, 3H, OAc), 1.88 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.0, 170.7, 170.4, 170.2, 170.1, 168.5, 139.3, 139.1, 138.8, 138.7, 138.6, 137.7, 128.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.3, 128.0, 127.8, 127.7,

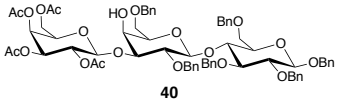
127.7, 127.6, 127.6, 127.5, 127.4, 127.3, 102.6 (C-1), 102.5 (C-1'), 98.5 (C-2''), 83.1 (C-6'), 82.0 (C-2), 78.6 (C-3), 76.6 (C-4), 76.5 (C-3'), 75.6 (Ph*C*H), 75.3 (C-2), 75.2 (Ph*C*H), 75.1 (Ph*C*H), 73.5 (Ph*C*H), 73.2 (Ph*C*H), 72.9 (H-6'), 72.6 (C-2'), 71.1 (Ph*C*H), 69.2 (C-4''), 68.9 (C-8''), 68.6 (C-6), 68.0 (C-4'), 67.3 (C-7''), 62.5 (C-9'') 62.4 (C-9''), 53.2 (O*C*H$_3$), 49.4 (C-5''), 36.6 (C-2''); HRMS calc. for $C_{74}H_{86}NO_{23}$ $[M+H]^+$: 1356.5591, found 1356.5457; HRMS calc. for $C_{74}H_{85}NO_{23}Na$ $[M+Na]^+$: 1378.5410, found 1378.5374.



39

(2R,3S,4R,5R,6S)-2-(acetoxymethyl)-6-(((2S,3R,4S,5S,6R)-3-(benzyloxy)-6-((benzyloxy) methyl)-5-hydroxy-2-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl) tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-5-(((2,2,2-trichloroethoxy) carbonyl)amino)tetrahydro-2H-pyran-3,4-diyldiacetate (39). A solution of **25** (1.5 eq., 299 mg, 0.479 mmol) and **11** (1.0 eq., 274 mg, 0.310 mmol) in CH_2Cl_2 (5.0 mL) was stirred over freshly activated 4 Å molecular sieves for 40 min. The solution was cooled to -5 °C and TMSOTf (1 drop) was added. The reaction stirred for 2 h, was quenched with triethylamine, filtered through a plug of Celite with additional CH_2Cl_2, washed with sat. aq. $NaHCO_3$ (2 x 10 mL), water (2 x 10 mL) and brine (1 x 10 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→1:2 hexanes/EtOAc) to yield **39** (192 mg, 46%) as a white solid: mp: 137-139 °C; R_f 0.45 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3058, 3031, 2869, 1751, 1231, 1072 ; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.21-7.41 (m, 30H, Ar), 5.00 (t, J=9.64 Hz, 1H, H-4"), 4.98 (d, J=10.72 Hz, 1H, PhC*H*), 4.92 (d, J=11.88 Hz, 1H, PhC*H*), 4.90 (d, J=10.72 Hz, 1H, PhC*H*), 4.89 (dd, J=9.32, 10.56 Hz, 1H, H-3"), 4.82 (d, J=12.08 Hz, 1H, PhC*H*), 4.77 (d, J=8.56 Hz, 1H, H-1"), 4.74 (d, J=10.60 Hz, 1H, PhC*H*), 4.73 (d, J=10.84 Hz, 1H,

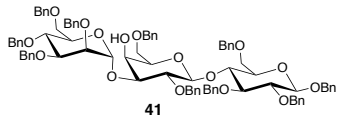
PhC*H*), 4.65 (d, J=12.12 Hz, 1H, PhC*H*), 4.63 (d, J=11.84 Hz, 1H, PhC*H*), 4.61 (d, J=11.36 Hz, 1H, PhC*H*), 4.54 (d, J=12.08 Hz, 1H, PhC*H*), 4.43-4.50 (m, 5H, PhC*H*, H-1, Troc-C*H*, H-1'), 4.37 (d, J=11.96 Hz, 1H, PhC*H*), 4.23 (dd, J=5.04, 12.36 Hz, 1H, H-6"), 4.12 (dd, J=2.24, 12.24 Hz, 1H, H-6"), 4.03 (t, J=9.32 Hz, 1H, H-4), 4.00 (brs, 1H, H-4'), 3.81 (dd, J=4.00, 10.96 Hz, 1H, H-6), 3.69-3.74 (m, 2H, H-6, H-6'), 3.42-3.66 (m, 8H, H-2', H-2", H-5", H-3', H-3, H-6', H-2, H-5'), 3.31-3.34 (m, 1H, H-5), 2.51 (brs, 1H, OH), 2.04 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.98 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 170.7, 170.6, 169.5, 139.1, 138.7, 138.5, 138.4, 137.6, 128.8, 128.5, 128.5, 128.4, 128.2, 127.0, 127.9, 127.8, 127.7, 127.7, 127.5, 127.4, 102.6 (C-1'), 102.4 (C-1), 101.0 (C-1"), 95.6 (Troc-*C*Cl_3), 83.0 (C-3), 81.9 (C-2), 80.7 (C-3'), 80.1 (C-2"), 75.7 (Ph*C*H), 75.3 (C-5), 75.2 (Ph*C*H), 75.0 (Troc-*C*H_2), 73.7 (Ph*C*H), 73.5 (Ph*C*H), 73.0 (C-5'), 72.0 (C-5"), 71.8 (C-3"), 71.1 (Ph*C*H), 68.6 (C-4"), 68.4 (C-6'), 68.3 (C-6), 68.2 (C-4'), 62.1 (C-6"), 56.3 (C-2"), 20.8 (OAc), 20.6 (OAc), 20.7 (OAc); HRMS calc. for $C_{69}H_{76}Cl_3NO_{20}Na$ $[M+Na]^+$: 1366.3924, found 1366.3805.



40

(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(((2S,3R,4S,5S,6R)-3-(benzyloxy)-6-((benzyloxy) methyl)-5-hydroxy-2-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl) tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (40). A solution of **32** (1.3 eq., 113 mg, 0.229 mmol) and **11** (1.0 eq., 160 mg, 0.181 mmol) in CH_2Cl_2 (4 mL) was stirred over freshly activated 4Å MS for 1 h. The solution was cooled to -20°C and TMSOTf (1 drop) was added. The reaction stirred for 2 h, was quenched with triethylamine, filtered through a plug of Celite with additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→3:2 hexanes/EtOAc)

to yield **39** (113 mg, 51%) as a white foam: mp: 56-58 °C; R_f 0.52 (1:1 EtOAc/hexanes); IR (thin film, cm^{-1}): 3016, 2857, 1744; ^1H NMR (400 MHz, $CDCl_3$): δ 7.42-7.22 (m, 30H, Ph), 5.38 (dd, 1H, J=0.76, 3.36 Hz, H-4"), 5.27 (dd, 1H, J=8.00, 10.44 Hz, H-2"), 5.00-4.96 (m, 2H, PhC*H*, H-3"), 4.93 (d, 1H, J=12.08 Hz, PhC*H*), 4.89 (d, 1H, J=10.88 Hz, PhC*H*), 4.78 (d, 1H, J=8.04 Hz, H-1"), 4.74-4.70 (m, 3H, PhC*H*), 4.64 (d, 1H, J=12.2 Hz, PhC*H*), 4.60 (d, 1H, J=11.28 Hz, PhC*H*), 4.59 (d, 1H, J=12.2 Hz, PhC*H*), 4.48 (d, 1H, J=7.32 Hz, H-1'), 4.47 (d, 1H, J=11.56 Hz, PhC*H*), 4.46 (d, 1H, J=7.4 Hz, H-1), 4.41 (d, 1H, J=12.2 Hz, PhC*H*), 4.36 (d, 1H, J=12.0 Hz, PhC*H*), 4.15-4.10 (m, 2H, H-6"), 4.03-3.98 (m, 2H, H-4, H-4'), 3.90 (t, 1H, J=6.8 Hz, H-5"), 3.77 (dd, 1H, J=4.28, 11.08 Hz, H-6), 3.72 (dd, 1H, J=7.2, 9.64 Hz, H-6'), 3.68 (dd, 1H, J=1.48, 10.88 Hz, H-6), 3.62-3.42 (m, 6H, H-2', H-3', H-3, H-6', H-2, H-5'), 3.32-3.28 (m, 1H, H-5), 2.54 (s, 1H, OH), 2.17 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.98 (s, 3H, Ac), 1.76 (s, 3H, Ac); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.6, 170.4, 170.2, 169.5, 139.2, 138.8, 138.6, 138.5, 138.4, 137.7, 128.5, 128.5, 128.4, 128.2, 128.2, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.5, 127.3, 102.7 (C-1), 102.3 (C-1'), 101.6 (C-1"), 83.0 (C-3), 82.4 (C-3'), 82.0 (C-2), 79.2 (C-2'), 76.4 (C-4'), 75.6 (Ph*C*H), 75.3 (C-5), 75.2 (Ph*C*H), 75.2 (Ph*C*H), 73.7 (Ph*C*H), 73.4 (Ph*C*H), 72.9 (C-5'), 71.1 (C-5"), 71.0 (Ph*C*H), 71.0 (C-3"), 69.0 (C-2"), 68.5 (C-6'), 68.3 (C-6), 68.1 (C-4), 67.1 (C-4"), 61.5 (C-6"), 20.8 (Ac), 20.7 (Ac), 20.6 (Ac); HRMS calc. for $C_{68}H_{76}O_{20}Na$ $[M+Na]^+$: 1235.4828, found 1235.5840.

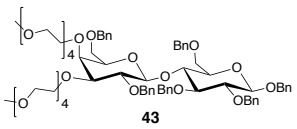


41

(2R,3S,4S,5R,6S)-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)-4-(((2R,3S,4S,5R,6R)-

3,4,5-tris(benzyloxy)-6-((benzyloxy) methyl)tetrahydro-2H-pyran-2-yl)oxy) tetrahydro-2H-pyran-3-ol (41). Mannosyl donor **34** and lactose acceptor **11** were dried on high vac for 12 h. A solution of **34** (162 mg, 0.184 mmol) and **11** (176 mg, 0.278 mmol) in Et_2O:CH_2Cl_2 (1:1, 12 mL) was stirred over freshly activated 4Å mol sieves for 1 h. The solution was cooled to -50 °C and NIS (81.9 mg, 0.364 mmol) and AgOTf (37.2 mg, 0.145 mmol) was added and the reaction mixture warmed to room temperature and stirred for 12 h. The reaction was quenched with Et_3N, filtered through a plug of Celite, washed with $Na_2S_2O_3$, water, and brine, dried over $MgSO_4$, concentrated *in vacuo*, and purified by medium pressure chromatography (CombiFlash EZ Prep, 12g Gold, 40 m, hexanes→1:4 hexanes/EtOAc) to yield **41** as a clear oil (100 mg, 39%): R_f: 0.51 (3:2 hexanes:EtOAc); IR (thin film, cm^{-1}): 2914, 2873; ^1H NMR (400 MHz, $CDCl_3$): δ 7.02-7.39 (m, 50H, Ar), 5.05 (d, J=1.48 Hz, 1H, H-1”), 4.98 (d, J=11.00 Hz, 1H, PhC*H*), 4.94 (d, J=11.00 Hz, 1H, PhC*H*), 4.90 (d, J=11.00 Hz, 1H, PhC*H*), 4.89 (d, J=11.00 Hz, 1H, PhC*H*), 4.81 (d, J=11.00 Hz, 1H, PhC*H*), 4.75 (d, J=11.00 Hz, 1H, PhC*H*), 4.72 (d, J=11.00 Hz, 1H, PhC*H*), 4.69 (d, J=11.00 Hz, 1H, PhC*H*), 4.65 (d, J=11.00 Hz, 1H, PhC*H*), 4.57-4.63 (m, 5H, PhC*H*), 4.51 (d, J=11.00 Hz, 1H, PhC*H*), 4.48 (d, J=7.76 Hz, 1H, H-1), 4.41-4.44 (m, 3H), 4.42 (d, J=7.04 Hz, 1H, H-1’), 4.35 (d, J=11.00 Hz, 1H, PhC*H*), 4.30 (d, J=11.00 Hz, 1H, PhC*H*), 3.98-4.05 (m, 4H, H-5”, H-4”, H-3, H-4’), 3.94 (dd, J=2.60, 9.76 Hz, 1H, H-3”), 3.83 (t, J=2.48 Hz, 1H, H-2”), 3.74 (dd, J=4.12, 10.84 Hz, 1H, H-6’), 3.57-3.69 (m, 5H, H-3’, H-6’, H-6”, H-6, H-6”), 3.55 (d, J=8.88 Hz, 1H, H-4), 3.45-3.50 (m, 3H, H-6, H-2’, H-2), 3.30-3.35 (m, 1H, H-5’), 2.29 (d, J=3.56 Hz, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.3, 139.0, 138.6, 138.5, 138.4, 138.1, 136.8, 128.6, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 102.7 (C-1, C-1’’), 93.6 (C-1”), 83.1 (C-4), 81.9 (C-2), 79.8 (C-3”), 78.1 (C-2’), 76.5 (C-3), 75.8 (Ph*C*H), 75.7 (C-3’), 75.5 (Ph*C*H), 75.3 (C-5’), 75.2 (C-2”), 75.2 (Ph*C*H), 75.1 (Ph*C*H), 75.1 (Ph*C*H), 75.0 (C-

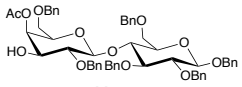
4”), 73.6 (Ph*C*H), 73.1 (Ph*C*H), 73.1 (Ph*C*H), 73.0 (Ph*C*H), 72.5 (C-5), 72.2 (Ph*C*H), 71.7 (C-5”), 71.1 (Ph*C*H), 69.1 (C-6”), 69.0 (C-6), 68.3 (C-6’), 64.9 (C-4”); HRMS calc. for $C_{88}H_{92}O_{16}Na$ $[M+Na]^+$: 1427.6283, found 1427.6210



43

(2R,3S,4S,5R,6S)-3,4-bis((2,5,8,11-tetraoxatridecan-13-yl)oxy)-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl) tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran (43). To a solution of lactose acceptor **11** (1.0 eq., 213 mg, 0.241 mmol) and **36** (2.6 eq, 225 mg, 0.620 mmol) in DMF (2.5 mL) at 0°C was added NaH (60%, 16 eq., 160 mg, 4.0 mmol). The reaction warmed to ambient temperature and stirred for 48 h. The reaction was quenched with water and extracted with EtOAc (3 x 15 mL). The organic extracts were combined and washed with water (2 x 10 mL) and brine (1 x 10 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude oil was purified by flash column chromatography (hexanes→EtOAc) to yield **43** (257 mg, 84%) as a clear residue: R_f 0.42 (EtOAc), IR (thin film, cm^{-1}): 3031, 2873; ^1H NMR (400 MHz, $CDCl_3$): δ 7.23-7.41 (m, 30H, Ar), 5.00 (d, J=10.64, 1H, PhC*H*), 4.94 (d, J=12.04, 1H, PhC*H*), 4.90 (d, J=10.84, 1H, PhC*H*), 4.79 (d, J=11.24, 1H, PhC*H*), 4.72 (d, J=11.88, 1H, PhC*H*), 4.71 (d, J=10.08, 1H, PhC*H*), 4.69 (d, J=11.24, 1H, PhC*H*), 4.65 (d, J=12.04, 1H, PhC*H*), 4.53 (d, J=12.16, 1H, PhC*H*), 4.71 (d, J=7.68, 1H, H-1), 4.42 (d, J=11.72, 1H, PhC*H*), 4.41 (d, J=7.40, 1H, H-1’), 4.40 (d, J=12.28, 1H, PhC*H*), 4.36 (d, J=11.72, 1H, PhC*H*), 4.02-4.06 (m, 1H), 3.92 (dd, J=9.16, 9.40, 1H, H-4), 3.71-3.82 (m, 5H), 3.51-3.68 (m, 32H), 3.46 (dd, J=7.72, 8.84, 1H, H-2), 3.38-3.42 (m, 1H, H-6’), 3.36 (s, 3H, OCH_3), 3.35 (s, 3H, OCH_3), 3.34-3.38 (m, 1H, H-5), 3.31-3.34 (m, 1H, H-5’), 3.23 (dd, J=2.92, 9.68, 1H, H-

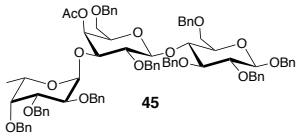
3'); ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.3, 139.1, 138.8, 138.6, 138.4, 137.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 102.9 (C-1'), 102.6 (C-1), 83.7 (C-3'), 83.1 (C-2), 82.0 (C-3), 79.9 (C-2'), 76.9 (C-4), 75.5, 75.3 (C-5), 75.2, 75.1, 74.5 (C-4'), 73.5, 73.2, 73.1 (C-5'), 72.6, 72.0, 71.1, 71.0, 70.9, 70.8, 70.7, 70.6, 70.4, 68.5 (C-6), 68.2 (C-6'), 59.2 (O*C*H$_3$); HRMS calc. for $C_{88}H_{92}O_{16}Na$ $[M+Na]^+$: 1285.6287, found 1285.6274.



44

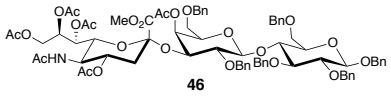
(2R,3R,4S,5R,6S)-5-(benzyloxy)-2-((benzyloxy)methyl)-4-hydroxy-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-3-yl acetate (44). To a solution of **11** (1.0 eq, 3.38 g, 3.83 mmol) in MeCN (40 mL, 0.1 M) was added trimethylorthoacetate (3.0 eq, 1.56 mL, 11.5 mmol) and TsOH (cat). The reaction stirred at room temperature for 120 min then 80% TFA (4.0 eq, 1.50 mL, 15.3 mmol) was added and the reaction stirred for 15 min. The reaction was quenched with trimethylamine, concentrated, and purified by flash column chromatography (4:1 hexanes/EtOAc) to yield axially acetylated lactose acceptor **44** (3.30 g, 93%) as a white foam: mp: 33-35 °C; R_f 0.45 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3441, 2868, 1742, 1095, 1058; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.16 (m, 30H, Ar), 5.33 (d, 1H, J=3.20 Hz, H-4'), 4.97 (d, J=10.6 Hz, 1H, PhC*H*), 4.95 (d, J=12.1 Hz, 1H, PhC*H*), 4.92 (d, J=10.9, 1H, PhC*H*), 4.80 (d, J=11.4 Hz, 1H, PhC*H*), 4.76 (d, J=9.08 Hz, 1H, PhC*H*), 4.66 (d, J=11.7 Hz, 1H, PhC*H*), 4.63 (d, J=12.3 Hz, 1H, PhC*H*), 4.49 (d, J=7.24 Hz, 1H, H-1), 4.47 (d, J=7.32 Hz, 1H, H-1'), 4.46 (d, J=11.7 Hz, 1H, PhC*H*), 4.45 (d, J=12.0 Hz, 1H, PhC*H*), 4.24 (d, J=12.0 Hz, 1H, PhC*H*), 4.03 (dd, J=9.16, 9.32 Hz, 1H, H-4), 3.82 (dd, J=4.04, 11.0 Hz, 1H, H-6), 3.75 (dd, J=1.72, 101.0 Hz, 1H, H-6), 3.63 (dd, J=3.48, 9.56 Hz, 1H, H-3'), 3.57 (dd, J=8.84, 9.08 Hz, 1H, H-3), 3.52-3.48 (m, 2H, H-5', H-2), 3.42-3.36 (m, 2H, H-2', H-5),

3.34 (d, J=6.64 Hz, 2H, H-6'), 2.03 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.1, 139.1, 138.7, 138.3, 138.0, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 102.6 (C-1), 102.3 (C-1'), 83.3 (C-3), 82.9 (C-2), 81.9 (C-2'), 80.2 (C-4), 75.5 (Ph*C*H), 75.3 (C-5), 75.2 (Ph*C*H), 75.2 (Ph*C*H), 73.5 (Ph*C*H), 73.4 (Ph*C*H), 72.6 (C-3'), 72.1 (C-5'), 71.1 (Ph*C*H), 69.7 (C-4'), 68.3 (C-6), 68.2 (C-6), 67.4 (C-6'), 20.9 (Ac); LRMS calc. for $C_{56}H_{64}O_{12}N$ $[M+Na]^+$: 942.4, found 942.6.



(2R,3S,4S,5R,6S)-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)-4-(((2S,3S,4R,5R,6S)-3,4,5-tris(benzyloxy)-6-methyltetrahydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3-yl acetate (45). A solution **44** (2.2 eq, 1.10 g, 2.50 mmol), **16** (1.0 eq, 1.07 g, 1.16 mmol), $SnCl_2$ (10.2 eq., 2.2309 g, 11.77 mmol), $AgClO_4$ (7.4 eq., 1.7772 g, 8.57 mmol), and 2,6-di-tert-butylpyridine (10 eq., 3.0 mL, 10.0 mmol) in Et_2O (0.1 M, 10 mL) was stirred over freshly activated 4Å MS for 72 h. The mixture was filtered through a plug of Celite using CH_2Cl_2 and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→30% EtOAc/hexanes) to yield **45** (1.23 g, 79%) as a white foam: mp: 35-37 °C; R_f 0.65 (1:1 hexanes:EtOAc); IR (thin film, cm^{-1}): 2912, 2852, 1740; ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.25 (d, J=3.60, 1H, H-1''), 4.96 (d, J=11.52, 1H, PhC*H*), 4.95 (d, J=10.86, 1H, PhC*H*), 4.92 (d, J=12.06, 1H, PhC*H*), 4.88 (d, J=10.86, 1H, PhC*H*), 4.83 (d, J=11.70, 1H, PhC*H*), 4.82 (d, J=11.88, 1H, PhC*H*), 4.73 (d, J=11.76, 1H, PhC*H*), 4.72 (d, J=10.80, 1H, PhC*H*), 4.71 (d, J=10.92, 1H, PhC*H*), 4.70 (d, J=11.88, 1H, PhC*H*), 4.64 (d, J=11.52, 1H, PhC*H*), 4.62 (d, J=12.06, 1H, PhC*H*),

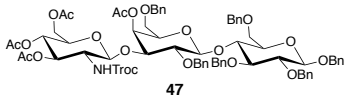
4.57 (d, J=12.18, 1H, PhC*H*), 4.56 (d, J=12.00, 1H, PhC*H*), 4.44 (d, J=12.06, 1H, PhC*H*), 4.43 (d, J=7.62 1H, H-1), 4.41 (d, J=7.38, 1H, H-1'), 4.39 (d, J=11.88, 1H, PhC*H*), 4.34 (d, J=12.12, 1H, PhC*H*), 4.07 (q, J=6.36, 1H, H-5''), 4.04 (dd, J=3.66, 10.2, 1H, H-4''), 3.97-4.00 (m, 2H, H-2'', H-4), 3.92 (d, J=2.64, 1H, H-4'), 3.75 (dd, J=3.96, 10.92, 1H, H-6), 3.70 (d, J=1.32, 1H, H-3''), 3.63 (dd, J=6.96, 9.84, 1H, H-6'), 3.44-3.61 (m, 6H, H-2', H-6, H-3', H-3, H-6', H-2), 3.34 (t, J=6.12, 1H, H-5'), 3.22 (ddd, J=1.68, 3.78, 9.90, 1H, H-5), 2.41 (s, 1H, O-H), 1.15 (d, J=6.48, 3H, H-6''); ^{13}C NMR (150 MHz, $CDCl_3$): δ 139.3, 139.1, 138.9, 138.8, 138.7, 138.6, 138.4, 138.3, 137.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.55 127.4, 127.3, 127.2, 127.1, 102.7 (C-1'), 102.6, (C-1), 99.4 (C-1''), 83.2 (C-3), 81.9 (C-2), 80.3 (C-3'), 79.3 (C-2''), 79.2 (C-2'), 77.8 (C-3''), 76.5 (C-4), 75.9 (C-4''), 75.5 (Ph*C*H), 75.1 (Ph*C*H), 75.1 (C-5), 75.0 (Ph*C*H), 74.6 (Ph*C*H), 73.6 (Ph*C*H), 73.1 (Ph*C*H), 72.8 (H-5'), 71.1 (Ph*C*H), 69.0 (C-4'), 68.6 (C-6'), 68.1 (C-6), 67.2 (C-5''), 16.9 (C-6''). LRMS calc. for $C_{83}H_{88}O_{16}K$ $[M+K]^+$: 1379.6, found 1378.9.



46

(1S,2R)-1-((2R,3R,4S,6S)-3-acetamido-4-acetoxy-6-(((2R,3S,4S,5R,6S)-3-acetoxy-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-2-yl)propane-1,2,3-triyl triacetate (46). A solution of **20** (1.0 eq., 50.8 mg, 0.069 mmol) and **44** (1.3 eq., 79.3 mg, 0.090 mmol) in proprionitrile (2.0 mL) was stirred over freshly activated 4Å MS for 30 min. The solution was cooled to -30 °C and trimethylsilyl trifluoromethanesulfonate (1 drop) was added. The reaction slowly warmed to ambient temperature and stirred for 24 h. The reaction was quenched with triethylamine, filtered

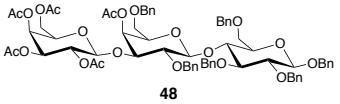
through a plug of Celite using additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (1:1 hexanes/EtOAC→ EtOAc) to yield **46** (42.1 mg, 44.9%) as a white solid: mp: 48-51 °C; R_f 0.41 (EtOAc); IR (thin film, cm^{-1}): 3384, 2924, 2853, 1746, 1227 ; ^1H NMR (400 MHz, $CDCl_3$): δ 7.41-7.16 (m, 30H, Ar), 5.62-5.58 (m, 1H, H-8''), 5.33 (dd, 3H, PhC*H*, H-4"), 4.89 (d, 1H, J=12.2 Hz, PhC*H*), 4.77 (d, 1H, J=7.36 Hz, H-1'), 4.75 (d, 1H, J=10.5 Hz, PhC*H*), 4.69 (d, 1H, J=10.9, PhC*H*), 4.64 (d, 1H, J=12.5 Hz, PhC*H*), 4.63 (d, 1H, J=12.0 Hz, PhC*H*), 4.53 (d, 1H, J=12.3 Hz, PhC*H*), 4.50 (dd, 1H, J=3.40, 9.48 Hz, H-4'), 4.44 (d, 1H, J=7.64, H-1), 4.40 (d, 1H, J=11.8 Hz, PhC*H*), 4.38 (d, 1H, J=11.7 Hz, PhC*H*), 4.30 (dd, 1H, J=2.32, 12.5 Hz, H-9"), 4.20 (d, 1H, J=11.8 Hz, PhC*H*), 4.13-4.05 (m, 1H, H-5"), 4.02-3.95 (m, 2H, H-9", H-4), 3.83 (s, 3H, O*CH*$_3$), 3.77 (dd, 1H, J=1.24, 11.3 Hz, H-6), 3.72 (dd, 1H, J=2.68, 10.7 Hz, H-6"), 3.68 (t, 1H, J=7.32 Hz, H-6'), 3.63 (dd, 1H, J=5.4, 11.1 Hz, H-6), 3.54 (t, 1H, J=9.00 Hz, H-3), 3.48-3.43 (m, 3H, H-2', H-3, H-2'), 3.35-3.28 (m, 2H, H-5, H-6'), 2.50 (dd, 1H, J=4.68, 12.6 Hz, H-3), 2.09 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.86 (s, 3H, Ac) 1.87-1.83 (m, 1H, H-3), 1.77 (s, 3H, Ac); ^{13}C NMR (100 MHz, $CDCl_3$): δ, 170.9, 170.7, 170.5, 170.1, 168.0, 139.6, 139.4, 138.8, 138.8, 138.3, 137.7, 129.3, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 127.2, 102.5 (C-1), 102.2 (C-1'), 97.4 (C-2"), 83.0 (C-3), 82.0 (C-2'), 79.7 (C-3'), 75.2 (C-4), 75.1 (C-5), 75.0 (Ph*C*H), 74.0 (Ph*C*H), 73.3 (C-4'), 72.3 (Ph*C*H), 71.5 (Ph*C*H), 71.0 (C-6"), 70.5 (Ph*C*H), 69.7 (Ph*C*H), 68.9 (C-6), 68.8 (C-5'), 68.5 (C-8''), 67.8 (C-6'), 67.2 (C-7"), 62.2 (C-9''), 53.2 (O*C*H$_3$), 52.7 (C-2'), 49.4 (C-5''), 37.7 (C-3''), 23.4, 21.4, 21.0, 20.9, 20.6; LRMS calc. for $C_{76}H_{87}NO_{24}NH_4$ $[M+NH_4]^+$: 1415.6, found 1415.7.



47

(2R,3S,4R,5R,6S)-6-(((2R,3S,4S,5R,6S)-3-acetoxy-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-2-(acetoxymethyl)-5-(((2,2,2-trichloroethoxy)carbonyl)amino)tetrahydro-2H-pyran-3,4-diyl diacetate (47). A solution of **25** (1.3 eq., 1.04 g, 1.67 mmol) and **44** (1.0 eq., 1.14 g, 1.24 mmol) in CH_2Cl_2 (12.5 mL) was stirred over freshly activated 4 Å molecular sieves for 60 min. The solution was cooled to -5 °C and TfOH (0.3 mL of solution of 1 drop in 1.0 mL CH_2Cl_2) was added. The reaction stirred for 15 min, was quenched with triethylamine, filtered through a plug of Celite with additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→1:2 hexanes/EtOAc) to yield **47** (1.58 g, 92%) as a white solid: mp: 52-54 °C; R_f 0.40 (1:1 hexanes/EtOAc); IR (thin film, cm^{-1}): 3397, 3054, 2987, 1752, 1712; ^1H NMR (400 MHz, $CDCl_3$): δ 7.42-7.16 (m, 30H, Ar), 5.41 (d, J=3.44 Hz, 1H, H-4'), 5.01 (dd, J=9.60, 9.64 Hz, 1H, H-4'), 4.98 (d, J=10.6 Hz, 1H, PhC*H*), 4.93 (d, J=12.2 Hz, 1H, PhC*H*), 4.90 (d, J=11.1 Hz, 1H, PhC*H*), 4.87 (d, J=11.9 Hz, 1H, PhC*H*), 4.74 (d, J=10.5, 1H, H-3"), 4.73 (d, J=11.0 Hz, 1H, PhC*H*), 4.70 (d, J=12.2 Hz, 1H, PhC*H*), 4.69-4.63 (m, 2H, C*H*$_2$CCl$_3$), 4.67 (d, J=9.68 Hz, 1H, H-1"), 4.58 (d, J=12.0 Hz, 1H, PhC*H*), 4.49 (d, J=12.0 Hz, 1H, PhC*H*), 4.48 (d, J=8.08 hZ, 1H, H-1'), 4.46 (d, J=7.76 Hz, 1H, H-1) 4.44 (m, 2H,), 4.37 (d, J=9.96 Hz), 4.27 (d, J=11.92 Hz, 2H, PhC*H*), 4.20 (d, J=3.44 Hz, 2H, H-6"), 4.05 (dd, J=9.28, 9.36 Hz, 1H, H-4), 3.80 (dd, J=3.84, 11.2 Hz, 1H, H-6), 3.72-3.64 (m, 2H, H-6, H-3'), 3.62-3.51 (m, 5H, H-3, H-2', H-5', H-2", H-5"), 3.47 (dd, J=7.72, 9.00 Hz, H-2), 3.34 (d, J=6.92 Hz, 2H, H-6'), 3.33-3.29 (m, 1H, H-5), 2.06 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.96 (s, 3H, OAc)**;** ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.9, 170.7, 170.0, 169.4, 154.0 139.1, 138.7, 138.3, 138.1, 137.6, 128.9, 128.6, 128.5, 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.5, 127.0, 102.6 (C-1), 102.1 (C-1'), 101.1 (C-1"),

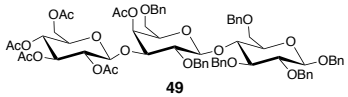
99.7 (Troc-*C*Cl_3), 82.8 (C-5”), 81.8 (C-2), 81.1 (C-2’), 76.0 (C-3’), 75.5 (C-4), 75.2 (Ph*C*H), 75.1 (C-5), 75.0 (Ph*C*H, Troc-*C*H_2), 74.4 (Ph*C*H), 73.7 (Ph*C*H), 73.6 (C-5’), 72.7 (C-3), 72.0 (Ph*C*H), 71.9 (C-3”), 71.1 (Ph*C*H), 69.6 (C-4’), 68.7 (C-4”), 68.1 (C-6), 68.0 (C-6’), 62.0 (C-6’), 56.2 (C-2”), 20.9 (OAc), 20.9 (OAc), 20.8 (OAc), 20.7 (OAc); LRMS calc. for $C_{75}H_{85}Cl_3N_3O_{21}$ $[M+2ACN+H]^+$: 1468.5, found 1468.3.



48

(2S,3R,4S,5S,6R)-2-(((2R,3S,4S,5R,6S)-3-acetoxy-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-6-(acetoxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (48). A solution of **28** (1.4 eq., 0.922 g, 1.87 mmol) and **44** (1.0 eq., 1.264 g, 1.34 mmol) in CH_2Cl_2 (10 mL, 0.1 M) was stirred over freshly activated 4 Å molecular sieves for 60 min. The solution was cooled to -5 °C and TfOH (0.300 mL of solution of 1 drop in 1.00 mL CH_2Cl_2) was added. The reaction stirred for 15 min, was quenched with triethylamine, filtered through a plug of Celite with additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→1:2 hexanes/EtOAc) to yield **48** (1.72 g, 95%) as a white foam: mp: 44-47°C; R_f 0.39 (1:1 EtOAc/hexanes); IR (thin film, cm^{-1}): 2870, 1751; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.19 (m, 30H, Ph), 5.40 (d, 1H, J=3.52 Hz, H-4’), 5.34 (dd, 1H, J=0.83, 3.45 Hz, H-4”), 5.14 (dd, 1H, J=7.88, 10.5 Hz, H-2”), 4.98 (d, 1H, J=10.6 Hz, PhC*H*), 4.96-4.92 (m, 2H, PhC*H*, H-3”), 4.90 (d, 1H, J=10.9 Hz, PhC*H*), 4.80 (d, 1H, J=7.88 Hz, H-1”), 4.74 (d, 1H, J=10.6 Hz, PhC*H*), 4.73 (d, 2H, J=10.9 Hz, PhC*H*), 4.65 (d, 1H, J=12.1 Hz, PhC*H*), 4.62 (d, 1H, J=10.3 Hz, PhC*H*), 4.62 (d, 1H, J=12.0 Hz, PhC*H*), 4.59 (d, 1H, J=10.7 Hz, PhC*H*), 4.47 (d, 1H, J=7.52 Hz, H-1), 4.47 (d, 1H, J=7.80 Hz, H-1), 4.43 (d, 1H, J=11.9 Hz, PhC*H*),

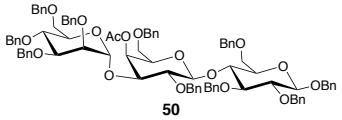
4.42 (d, 1H, J=12.2 Hz, PhC*H*), 4.29 (d, 1H, J=11.8 Hz, PhC*H*), 4.15 (dd, 1H, J=1.92, 6.48 Hz, H-6"), 4.02 (dd, 1H, J=9.16, 9.36 Hz, H-4), 3.83 (dt, 1H, J=0.71, 6.68 Hz, H-5"), 3.76 (dd, 1H, J=4.08, 11.08 Hz, H-6), 3.71-3.67 (m, 2H, H-3',H-6), 3.56-3.45 (m, 4H, H-3, H-2', H-5', H-2), 3.35 (d, 2H, J=6.20 Hz, H-6'), 3.33-3.29 (m, 1H, H-5), 2.17 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.86 (s, 3H, Ac); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.6, 170.5, 170.3, 169.9, 169.2, 139.2, 138.7, 138.4, 138.3, 138.2, 137.7, 128.6, 128.5, 128.3, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5, 102.6 (C-1'), 102.2 (C-1), 101.0 (C-1"), 82.8 (C-3), 81.9 (C-2), 80.4 (C-2'), 77.7 (C-3'), 76.1 (C-4), 75.4 (Ph*C*H), 75.3 (Ph*C*H), 75.2 (C-5), 75.1 (Ph*C*H), 73.7 (Ph*C*H), 73.4 (C-5'), 72.8 (Ph*C*H), 71.1 (Ph*C*H), 71.0 (C-3"), 70.8 (C-5"), 69.6 (C-4'), 69.4 (C-2"), 69.3 (C-6), 67.2 (C-6'), 67.1 (C-4"), 61.2 (C-6"), 20.9 (Ac), 20.8 (Ac), 20.7 (Ac); LRMS calc. for $C_{70}H_{82}NO_{21}$ $[M+NH_4]^+$: 1272.5, found 1272.9.



49

(2S,3R,4S,5R,6R)-2-(((2R,3S,4S,5R,6S)-3-acetoxy-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-6-(acetoxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (49). A solution of **31** (1.7 eq., 1.00 g, 2.03 mmol) and **44** (1.0 eq., 1.10 g, 1.19 mmol) in CH_2Cl_2 (10.0 mL, 0.1 M) was stirred over freshly activated 4 Å molecular sieves for 60 min. The solution was cooled to -5 °C and TfOH (0.300 mL of solution of 1 drop in 1.00 mL CH_2Cl_2) was added. The reaction stirred for 30 min, was quenched with triethylamine, filtered through a plug of Celite with additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→1:2 hexanes/EtOAc) to yield **49** (1.11 g, 75%) as a white foam: mp: 44-47 °C; R_f 0.50 (1:1 EtOAc/hexanes); IR (thin film, cm^{-1}): 3400, 2923, 1754; ^{1}H

NMR (400 MHz, $CDCl_3$): δ 7.39-7.17 (m, 30 H, Ph), 5.41 (d, 1H, J=3.52 Hz, H-4'), 5.16 (t, 1H, J=9.32 Hz, H-3"), 5.09 (dd, 1H, J=9.40, 9.60 Hz, H-4"), 5.01-4.98 (m, 2H, H-2", PhC*H*), 4.95 (d, 1H, J=11.8 Hz, PhC*H*), 4.91 (d, 1H, J=10.9 Hz, PhC*H*), 4.81 (d, 1H, J=7.80 Hz, H-1"), 4.74 (d, 1H, J=10.9 Hz, PhC*H*), 4.73 (d, 1H, J=10.9 Hz, PhC*H*), 4.71 (d, 1H, J=11.2 Hz, PhC*H*), 4.66 (d, 1H, J=12.1 Hz, PhC*H*), 4.62 (d, 1H, J=12.1 Hz, PhC*H*), 4.60 (d, 1H, J=10.7 Hz, PhC*H*), 4.48 (d, 1H, J=7.56 Hz, H-1), 4.46 (d, 1H, J=7.72 Hz, H-1'), 4.44, (d, 1H, J=11.1 Hz, PhC*H*), 4.41 (d, 1H, J=11.6 Hz, PhC*H*), 4.27 (d, 1H, J=11.8 Hz, PhC*H*), 4.24 (dd, 1H, J=2.88, 12.5 Hz, H-6"), 4.18 (dd, 1H, J=4.40, 12.1 Hz, H-6"), 4.02 (dd, 1H, J=9.24, 9.32 Hz, H-4), 3.77 (dd, 1H, J=4.16, 11.1 Hz, H-6), 3.70-3.62 (m, 3H, H-6, H-3', H-5"), 3.57-3.45 (m, 4H, H-3, H-5', H-2', H-2), 3.35 (d, 2H, J=6.28 Hz, H-6'), 3.35-3.31 (m, 1H, H-5), 2.07 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.84 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 170.4, 169.8, 169.5, 169.1, 139.2, 138.6, 138.4, 138.2, 138.2, 137.6, 128.6, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5, 102.6 (C-1), 102.1 (C-1'), 100.7 (C-1"), 82.8 (C-3), 81.9 (C-2), 80.0 (C-2'), 78.3 (C-3'), 76.2 (C-4), 75.5 (Ph*C*H), 75.4 (Ph*C*H), 75.3 (Ph*C*H), 75.2 (C-5), 73.7 (Ph*C*H), 73.4 (Ph*C*H), 73.0 (C-3"), 72.7 (C-5'), 71.8 (C-5"), 71.8 (C-2"), 71.1 (Ph*C*H), 69.7 (C-4'), 68.5 (C-4"), 68.2 (C-6, C-6'), 61.8 (C-6"), 20.8 (Ac), 20.7 (Ac), 20.6 (Ac); LRMS calc. for $C_{70}H_{82}NO_{21}$ $[M+NH_4]^+$: 1272.5, found 1272.8.

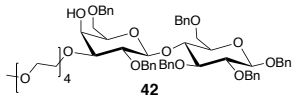


50

(2R,3S,4S,5R,6S)-5-(benzyloxy)-2-((benzyloxy)methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)-4-(((2R,3S,4S,5R,6R)-3,4,5-tris(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)tetrahydro-2H-

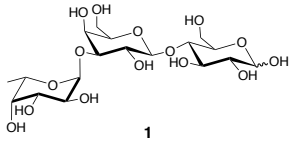
pyran-3-yl acetate (50). A solution of **34** (1.3 eq., 1.04 g, 1.65 mmol) and **44** (1.0 eq., 1.01 g, 1.09 mmol) in CH_2Cl_2 (10 mL, 0.1 M) was stirred over freshly activated 4 Å molecular sieves for 60 min. The solution was cooled to -5 °C and NIS (2.1 eq, 0.514 g, 2.28 mmol) and AgOTf (1.6 eq, 0.449 g, 1.75 mmol) were added. The reaction stirred for 15 min, was quenched with triethylamine, filtered through a plug of Celite with additional CH_2Cl_2, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (hexanes→1:2 hexanes/EtOAc) to yield **50** (1.471 g, 93%) as a white foam: mp: 35-38 °C; R_f 0.71 (1:1 EtOAc/hexanes); IR (thin film, cm^{-1}): 3063, 3030, 2923, 2855, 1741, 1453; ^1H NMR (400 MHz, $CDCl_3$): δ 7.53-6.99 (m, 50H, Ph), 5.46 (d, 1H, J= 3.08 Hz, H-4'), 5.32 (d, 1H, J=1.08 Hz, H-1"), 4.96 (d, 1H, J=10.3 Hz, PhC*H*), 4.94 (d, 1H, J=11.6 Hz, PhC*H*), 4.92 (d, 1H, J=10.3 Hz, PhC*H*), 4.91 (d, 1H, J=10.9 Hz, PhC*H*), 4.84 (d, 1H, J=12.5 Hz, PhC*H*), 4.73 (d, 2H, J=10.8 Hz, PhC*H*), 4.69 (d, 1H, J=12.7 Hz, PhC*H*), 4.66 (d, 1H, J=12.3 Hz, PhC*H*), 4.59 (d, 1H, J=10.2 Hz, PhC*H*), 4.58 (d, 1H, J=12.5 Hz, PhC*H*), 4.55 (d, 1H, J=10.2 Hz, PhC*H*), 4.49 (d, 1H, J=12.2 HZ, PhC*H*), 4.49 (d, 1H, J=10.4 Hz, PhC*H*), 4.49 (m, 2H, PhC*H*), 4.48 (d, 1H, J=6.44 Hz, H-1), 4.46 (d, 1H, J=7.60 Hz, H-1'), 4.42 (d, 1H, J=12.1 Hz, PhC*H*), 4.32 (d, 1H, J=12.4 Hz, PhC*H*), 4.28 (d, 1H, J=12.1 Hz, PhC*H*), 4.26 (d, 1H, J=12.2 Hz, PhC*H*), 4.04-3.96 (m, 3H, H-5", H-4, H-4"), 3.83 (dd, 1H, J=2.32, 2.92 Hz, H-3"), 3.81 (dd, 1H, J=3.28, 3.48 Hz, H-3'), 3.73 (dd, 1H, J=4.08, 11.0 H, H-6), 3.66-3.52 (m, 5H, H-6, H-6", H-2", H-3), 3.49 (d, 1H, J=7.6 Hz, H-2), 3.43 (dd, 1H, J=5.8, 6.84 HZ, H-5'), 3.39-3.31 (m, 4H, H-2', H-5, H-6'), 2.00 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.2, 139.1, 138.8, 138.7, 138.6, 138.5, 138.1, 137.8, 137.6, 129.0, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 103.0 (C-1), 102.7 (C-1'), 94.0 (C-1"), 83.2 (C-3), 82.2 (C-2), 79.5 (C-3"), 78.9 (C-2'), 77.6 (Ph*C*H), 76.4 (C-4), 76.2 (Ph*C*H), 75.8 (Ph*C*H), 75.5 (C-5), 75.4 (Ph*C*H), 75.4 (Ph*C*H), 75.2 (C-4"), 74.6 (C-2"), 74.2 (C-3'), 73.7 (Ph*C*H), 73.4 (Ph*C*H), 73.2

(Ph*C*H), 72.1 (Ph*C*H), 71.9 (Ph*C*H), 71.8 (C-5"), 71.7 (C-5'), 71.4 (Ph*C*H), 69.4 (C-6"), 68.5 (C-6), 67.3 (C-6'), 66.1 (C-4'), 21.2 (Ac); LRMS calc. for $C_{90}H_{94}O_{17}$ $[M+2Na]^{2+}$: 746.3, found 746.4.



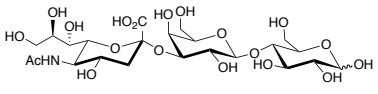
42

(2R,3S,4S,5R,6S)-3,4-bis((2,5,8,11-tetraoxatridecan-13-yl)oxy)-5-(benzyloxy)-2-((benzyloxy) methyl)-6-(((2R,3R,4S,5R,6R)-4,5,6-tris(benzyloxy)-2-((benzyloxy) methyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran (42). To a solution of lactose acceptor **44** (1.0 eq., 213 mg, 0.241 mmol) and **36** (2.6 eq, 225 mg, 0.620 mmol) in DMF (2.5 mL) at 0 °C was added NaH (60%, 16 eq., 0.160 g, 4.0 mmol). The reaction warmed to ambient temperature and stirred for 48 h. The reaction was quenched with water and extracted with EtOAc (3 x 15 mL). The organic extracts were combined and washed with water (2 x 10 mL) and brine (1 x 10 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude oil was purified by flash column chromatography (hexanes→EtOAc) to yield **42** (257 mg, 84%) as a clear oil: R_f 0.28 (EtOAc), IR (thin film, cm^{-1}): 3030, 2872, 1752, 1454; ^1H NMR (400 MHz, $CDCl_3$): δ 7.41-7.21 (m, 30H, Ar), 4.99 (dd, 1H, J=8.68, 10.4 Hz, PhC*H*), 4.94 (d,1H, J=12.0 Hz, PhC*H*), 4.90 (dd, 1H, J=2.96, 10.8 Hz, PhC*H*), 4.84 (d, 1H, J=11.24 Hz, PhC*H*), 4.75-4.70 (m, 3H, PhC*H*), 4.65 (d,1H, J=12.0 Hz, PhC*H*), 4.54 (dd, J=7.12, 12.2 Hz, 1H, PhC*H*), 4.50-4.37 (m, 4H), 3.99-3.91 (m, 1H), 3.85-3.32 (m, 31H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.4, 139.1, 138.8, 138.1, 137.7, 128.5, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 102.8, 102.6, 83.1, 82.0, 81.5, 78.2, 75.4, 75.3, 75.2, 75.1, 74.5, 73.6, 73.5, 73.2, 73.0, 72.0, 71.1, 70.7, 70.6, 70.5, 68.8, 68.5, 67.9, 59.1. LRMS calc. for $C_{63}H_{80}NO_{15}$ $[M+NH_4]^+$: 1090.6, found 1090.5.



1

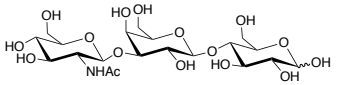
2R,3R,4R,5S,6R)-5-(((2S,3R,4S,5S,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-(((2S,3S,4R,5S,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-2-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-2,3,4-triol (1). To a solution of **45** (1.0 eq., 1.120 g, 0.835 mmol) in MeOH (50 mL) was added a concentrated solution of NaOMe (0.5 mL). The reaction stirred at ambient temperature for 4 h and was quenched with Dowex50Wx8 resin and filtered. $Pd(OH)_2/C$ (20%, 3.0 eq., 1.87 g, 2.7 mmol) was added to the resulting solution. The mixture was sparged with Ar then H_2 gas and the reaction stirred under an atmosphere of hydrogen for 24 h, was filtered through a plug of Celite, and concentrated *in vacuo*. The resulting residue was purified by P-2 Gel size exclution chromatography (H_2O) to yield **1** (325 mg, 80%) as a white solid: $[\alpha]^{23}_D$ -16.6° (*c* 10 mg/mL, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 5.23 (d, J=3.66 Hz, H-1α), 5.18 (d, J=3.72, H-1”), 4.67 (d, J=7.92 Hz, H-1β), 4.52 (d, J=6.75 Hz, H-1’), 4.18 (q, J=6.46 Hz, H-5”), 4.02 (br) 3.89-3.58 (m), 3.29 (t, J=8.4), 1.21 (d, J=6.6 Hz, H-6”); ^{13}C NMR (150 MHz, D_2O): δ 102.7 (C-1’), 100.9 (C-1”), 95.7 (C-1α), 91.8 (C-1β), 80.3, 78.4, 78.3, 75.3, 74.8, 74.4, 73.8, 71.7, 71.4, 71.1, 70.4, 70.1, 69.4, 68.6, 68.4, 67.1, 60.9, 60.0, 59.9, 15.3 (C-6”); HRMS calc. for $C_{18}H_{32}O_{15}Na$ $[M+Na]^+$: 511.1639, found 511.2829.



2

(2S,4S,5R,6R)-5-acetamido-2-(((2R,3S,4S,5R,6S)-3,5-dihydroxy-2-(hydroxymethyl)-6-(((2R,3S,4R,5R,6R)-4,5,6-trihydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-4-yl)oxy)-4-hydroxy-6-((1R,2R)-1,2,3-

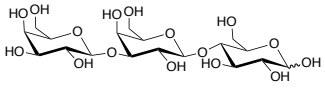
trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (2). To a solution of trisaccharide **46** (0.0671 g, 0.062 mmol) in MeOH (5 mL) was added a concentrated solution of NaOMe (0.1 mL). The reaction stirred at ambient temperature for 36 h and then water (2 mL) was added. Stirring at ambient temperature for an additional 48 h was followed by neturalization with Dowex50Wx8 resin, filtration with MeOH, and concentration. The crude material was resuspended in MeOH (50 mL) and Pearlman's catalyst (0.130 g, 4 eq, 0.25 mmol) was added. The reaction vessel was purged with Ar then H_2. The reaction stirred under H_2 for 24 h, was purged with Ar, filtered through a plug of Celite, concentrated, and purified by P-2 Gel (H_2O elutent) to yield **2** (0.0304 g, 78%) as a white solid: $[\alpha]^{23}_D$ +10.8° (*c* 10 mg/mL, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 5.23 (d, 0.35H, J=3.80 Hz, H-1α), 4.67 (d, 0.65H, J=7.96 Hz, H-1β), 4.54 (d, 1H, J=7.84 Hz, H-1'), 4.12 (dd, 1H, J=3.08, 9.88 Hz), 4.00-3.56 (m, 18H), 3.29 (t, J=8.16 Hz), 2.77 (dd, 1H, J=4.72, 12.6 Hz, H-3"eq), 2.04 (s, 3H, Ac), 1.81 (t, 1H, J=12.0 Hz, H-3"ax); ^{13}C NMR (150 MHz, D_2O): δ 175.2, 170.1 (CO_2H), 102.9, 102.6, 98.5 (C-2"), 95.7, 91.8, 78.4, 78.3, 78.1, 75.9, 75.4, 75.3, 75.2, 75.1, 74.9, 74.8, 74.3, 73.8, 73.7, 73.2, 72.9, 72.5, 71.5, 71.4, 71.1, 70.9, 70.6, 70.4, 70.2, 70.1, 69.7, 69.3, 68.5, 68.1, 68.1, 67.6, 67.5, 67.2, 66.8, 63.1, 63.0, 62.7, 61.0, 60.9, 60.8, 60.1, 59.9, 53.5, 52.0, 51.9, 51.6, 51.5, 39.4, 38.9, 38.8, 35.8, 22.0; HRMS calc. for $C_{38}H_{39}NO_{19}Na$ $[M+Na]^+$: 656.2014, found 656.3000.



3

N-((2S,3R,4R,5S,6R)-2-(((2R,3S,4S,5R,6S)-3,5-dihydroxy-2-(hydroxymethyl)-6-(((2R,3S,4R,5R,6R)-4,5,6-trihydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)oxy) tetrahydro-2H-pyran-4-yl)oxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (3). Activated Zn/PbO couple was added to a solution of **47** (0.729 g, 0.525 mmol)

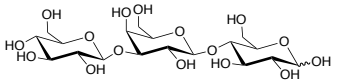
in THF:Ac_2O (10:1, 5.25 mL). The reaction was allowed to stir at ambient temperature under Argon atmosphere for 48 h. The reaction mixture was filtered and concentrated *in vacuo* to a clear residue. The crude product was resuspended in MeOH (15 mL) and NaOMe (0.5 mL) was added. After stirring at ambient temperature for 3 h, the reaction was quenched with Dowex50w8 resin, filtered, and concentrated *in vacuo* to a white crystalline solid. The solid was then dissolved in MeOH (20 mL) and $Pd(OH)_2$ (2.0 eq. 0.800 g, 1.0 mmol) was added. The reaction mixture was purged with Ar then H_2. The reaction stirred at ambient temperature for 48 h, was purged with Ar, filtered through a plug of Celite, concentrated, and purified by P-2 Gel (H_2O elutent) to yield **3** (0.234 g, 82%) as a white solid: $[\alpha]^{23}_D$ +5.3° (*c* 10 mg/mL, H_2O) ;^{1}H NMR (600 MHz, D_2O): δ 5.23 (d, J=.372 Hz, 0.65H, H-1α), 4.72 (d, J=8.58 Hz, 0.37H, H-1β), 4.71 (d, J=8.34 Hz, 1H, H-1”), 4.68 (d, J=7.98 Hz, 1H, H-1’), 4.45 (d, J=7.98 Hz, 1H), 4.10 (d, J=2.38 Hz, 1H), 3.97-3.39 (m), 3.28 (t, J=8.40 Hz, 0.7H), 2.07 (s, 3H, NHAc); ^{13}C NMR (150 MHz, D_2O): δ 174.7, 102.9, 102.2, 95.7, 91.8, 81.9, 78.3, 76.3, 75.5, 74.8, 74.4, 74.3, 73.8, 73.7, 73.5, 72.4, 71.1, 70.1, 69.9, 69.67 61.0, 60.9, 60.7, 60.0, 55.7, 22.3; HRMS calc. for $C_{18}H_{32}NO_{16}Na$ $[M+Na]^+$: 568.1854, found 568.3058.



4

(2R,3R,4R,5S,6R)-5-(((2S,3R,4S,5S,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-(((2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy) tetrahydro-2H-pyran-2-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-2,3,4-triol (4). To a solution of **48** (1.0 eq., 0.472 g, 0.376 mmol) in MeOH (10 mL) was added a concentrated solution of NaOMe (0.1 mL). The reaction stirred at ambient temperature for 3 h. The mixture was quenched with Dowex50w8, filtered, and concentrated *in vacuo*. The resulting white solid was

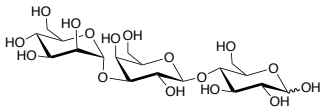
dissolved in MeOH (20 mL) and $Pd(OH)_2$ (3.0 eq., 0.790 g, 1.13 mmol) was added. The reaction was sparged with Ar then H_2. The reaction stirred under H_2 for 24 h and was purged with Ar, filtered through a plug of Celite and concentrated *in vacuo*. The crude solid was purified by P-2 Gel (H_2O elutent) to yield **4** (0.171 g, 90%) as a white solid: $[\alpha]^{23}_D$ +23.5° (*c* 10 mg/mL, H_2O); ^1H NMR (600 MHz, D_2O): δ 5.23 (br, 0.37H, H-1α), 4.67 (d, J=7.44 Hz, 0.9H, H-1β), 4.62 (d, J=6.96 Hz, 1H, H-1"), 4.51 (d, J=7.02 Hz, 1H, H-1'), 4.20 (br, 1H, H-4'), 3.97-3.60 (m), 3.29 (t, J=7.74 Hz, 0.5H); ^{13}C NMR (150 MHz, D_2O): δ 105.3, 102.5, 95.8, 91.8, 81.9, 78.2, 78.1, 75.0, 74.9, 74.8, 74.3, 73.8, 72.5, 71.4, 71.1, 71.0, 70.2, 70.1, 68.6, 68.4, 60.9, 60.1, 59.9; HRMS calc. for $C_{18}H_{32}O_{16}Na$ $[M+Na]^+$: 527.1588, found 527.2698



5

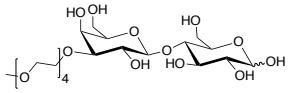
(2R,3R,4R,5S,6R)-5-(((2S,3R,4S,5S,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy) tetrahydro-2H-pyran-2-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-2,3,4-triol (5). To a solution of **49** (1.0 eq., 0.553 g, 0.441 mmol) in MeOH (10 mL) was added a concentrated solution of NaOMe (0.1 mL). The reaction stirred at ambient temperature for 3 h then quenched with Amberlyst Resin, filtered, and concentrated *in vacuo*. The resulting white solid was dissolved in MeOH (20 mL) and $Pd(OH)_2$ (3.0 eq., 0.93 g, 1.32 mmol) was added. The reaction was sparged with Ar then H_2. The reaction stirred under H_2 for 24 h and was sparged with Ar, filtered through a plug of Celite, and concentrated. The crude solid was purified by P-2 Gel (H_2O elutent) to yield **5** (0.207 g, 93%) as a white solid: $[\alpha]^{23}_D$ +37.2° (*c* 1 mg/mL, H_2O); ^1H NMR (400 MHz, D_2O): δ 5.23 (d, 0.39H, J=3.76 Hz, H-1α), 4.69 (d, 1H, J=7.80 Hz, H-1"), 4.68 (d, 0.61H, J=7.96 Hz, H-

1β), 4.52 (d, 1H, J=7.80 Hz, H-1'), 4.20 (d, 1H, J=3.12 Hz, H-4'), 3.99-3.28 (m); ^{13}C NMR (100 MHz, D_2O): δ 103.7, 102.4, 95.7, 91.7, 81.9, 78.1, 78.0, 75.7, 75.4, 74.9, 74.7, 74.3, 73.7, 73.2, 71.3, 71.1, 70.8, 70.6, 70.5, 70.0, 69.3, 68.2, 60.9, 60.6, 60.4, 60.0, 59.9, 48.8; LRMS calc. for $C_{18}H_{32}O_{16}Na$ $[M+Na]^+$: 527.2, found 527.4.



6

(2R,3R,4R,5S,6R)-5-(((2S,3R,4S,5S,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-(((2R,3S,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-2-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-2,3,4-triol (6). To a solution of **49** (1.0 eq., 1.471 g, 1.02 mmol) in MeOH (25 mL) was added a concentrated solution of NaOMe (0.1 mL). The reaction stirred at ambient temperature for 3 h and was then quenched with Amberlyst Resin, filtered, and concentrated *in vacuo*. The resulting white solid was dissolved in MeOH (30 mL) and $Pd(OH)_2$ (2.0 eq., 1.40 g, 2.03 mmol) was added. The reaction was sparged with Ar then H_2. The reaction stirred under H_2 for 24 h and was sparged with Ar, filtered through a plug of Celite, and concentrated. The crude solid was purified by P-2 Gel (H_2O elutent) to yield **6** (0.231 g, 45%) as a white solid: $[\alpha]^{23}_D$ +1.48° (*c* 0.5 mg/mL, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 5.23 (d, 0.4H, J=3.76 Hz, H-1α), 5.05 (d, 1H, J=1.28 Hz, H-1"), 4.69 (d, 0.6H, J=7.96 Hz, H-1β), 4.51 (d, 1H, J=7.80 Hz, H-1'), 4.19 (d, 1H, J=3.12 Hz, H-4'), 4.01-3.57 (m), 3.35 (s, 1H), 3.29 (t, 1H, J=8.72 Hz); ^{13}C NMR (150 MHz, D_2O): δ 102.8, 101.3, 96.2, 95.7, 81.1, 78.7, 78.5, 76.2, 76.1, 75.1, 75.0, 74.7, 74.4, 74.3, 73.8, 72.8, 72.7, 70.6, 70.3, 70.2, 69.5, 66.7, 66.6, 64.2, 61.0, 60.9, 60.1, 60.0; LRMS calc. for $C_{18}H_{32}O_{16}K$ $[M+K]^+$: 543.1, found 543.4



7

(2R,3R,4R,5S,6R)-5-(((2S,3R,4R,5S,6R)-4,5-bis((2,5,8,11-tetraoxatridecan-13-yl)oxy)-3-hydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-2,3,4-triol (7). Hydrogen gas was bubbled through a suspension of **42** (1.0 eq., 0.585 g, 0.552 mmol) and $Pd(OH)_2/C$ (20%, 2.0 eq., 0.780 g, 1.11 mmol) in MeOH/AcOH/acetone (1:1:1, 40 mL). The reaction stirred under atmosphere of hydrogen for 24 h, was filtered through a plug of Celite, and concentrated *in vacuo*. The crude material was purified by P-2 Gel (H_2O elutent) to yield **7** (0.226 g, 79%) as an opaque solid: $[\alpha]_D^{23}$ +41.4 (*c* 1 mg/mL, H_2O); ^1H NMR (400 MHz, D_2O): δ 5.21 (d, J=2.68 Hz, 0.55H, H-1α), 4.65 (d, J=7.72 Hz, 1H), 4.47 (d, J=7.64 Hz, 1H, H-1'), 4.42 (d, 0.45H, J=7.68 Hz, H-1β), 4.17 (d, 1H, J=1.64 Hz), 4.96-3.47 (m), 3.37 (s, 3H, OC*H*$_3$), 3.15 (t, J=5.28 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ 102.6, 102.5, 95.6, 91.8, 90.8, 75.2, 75.1, 71.3, 71.2, 70.9, 70.0, 69.9, 69.8, 69.5, 69.4, 69.3, 68.1, 61.0, 57.9; LRMS calc. for $C_{21}H_{40}O_{15}Na$ $[M+Na]^+$: 555.2, found 555.5.

Table 4. 9 Comparison of ^{1}H NMR spectroscopic data of 3'-fucosyllactose **(1)**

Reference[59] δ ^{1}H [ppm; mult; *J* (Hz)]	**Synthetic** δ ^{1}H [ppm; mult; *J* (Hz)] 600 MHz	**Deviation** (reference-synthetic) Δδ (ppm)
5.23; d; 3.96	5.23; d; 3.66	0
5.18; d; 3.96	5.18; d; 3.72	0
4.68; d; 8.25	4.67; d; 7.92	0.01
4.52; t	4.52; d; 6.75	0
-	4.18; q; 6.46	-
-	4.02; br	-
1.26	1.21; d; 6.6	0.05

Table 4. 10 Comparison of ^{13}C NMR spectroscopic data of 3'-fucosyllactose **(1)**

Reference[59] δ ^{13}C (ppm)	**Synthetic** δ ^{13}C (ppm) 150 MHz	**Deviation** (reference - synthetic) Δδ (ppm)
105.33	102.68	2.65
103.74	100.90	2.84
98.6	95.74	2.86
94.64	91.80	2.85
83.16	80.31	2.85
81.31	78.41	2.90
81.19	78.28	2.91
78.09	75.25	2.84
77.61	74.76	2.85
77.23	74.36	2.87
76.64	73.78	2.86
74.59	71.74	2.85
74.29	71.42	2.87
73.98	71.11	2.87
73.24	70.38	2.86
72.92	70.06	2.86
72.24	69.37	2.87
71.46	68.59	2.87
71.27	68.40	2.87
69.99	67.14	2.85
63.77	60.91	2.86
62.93	60.04	2.89
62.8	59.91	2.89
18.17	15.29	2.88

Table 4. 11 Comparison of ^{1}H NMR spectroscopic data of 3'-sialyllactose **(2)**

Reference[60] δ ^{1}H [ppm; mult; *J* (Hz)] 250 MHz	**Synthetic** δ ^{13}C (ppm) 600 MHz	**Deviation** (reference - synthetic) Δδ (ppm)
5.21; d; 3.7	5.22; br	0.01
4.65; d; 7.9	4.67; d; 7.62	-0.02
4.51; d; 7.8	4.53; d; 7.44	-0.02
4.10; d; br	4.13; m	-0.03
3.95-3.54; overlapped	3.96-3.59; m	-0.01
3.30; m	3.31; m	-0.01
2.74; dd; 4.4, 12.1	2.77; m	-0.03
2.04; s	2.04; s	0
1.83; t; 12.1	1.84; t; 12.06	-0.01

Table 4. 12 Comparison of ^{13}C NMR spectroscopic data of 3'-sialyllactose **(2)**

Reference[60] δ ^{13}C (ppm)	**Synthetic** δ ^{13}C (ppm) 150 MHz	**Deviation** (reference - synthetic) Δδ (ppm)
175.32	175.16	0.16
173.2	170.05	3.15
102.9	102.88	0.02
-	102.58	-
99.66	98.54	1.12
96.09	95.74	0.35
-	91.79	-
78.42	78.40	0.02
-	78.28	-
-	78.14	-
75.75	75.88	-0.13
75.39	75.44	-0.05
-	75.32	-
-	75.25	-
-	75.08	-
-	74.86	-
74.63	74.77	-0.14
74.11	74.31	-0.20
-	73.78	-
-	73.65	-
73.28	73.17	0.11
-	72.94	-
-	72.49	-
71.75	71.48	0.27
71.45	71.37	0.08
-	71.12	-
-	70.93	-

-	70.59	-
70.39	70.41	-0.02
-	70.21	-
-	70.06	-
69.69	69.66	0.03
-	69.34	-
68.33	68.52	-0.19
-	68.09	-
-	68.06	-
67.84	67.61	0.23
-	67.49	-
-	67.20	-
-	66.75	-
-	63.11	-
63.02	63.06	-0.04
-	62.66	-
61.3	61.00	0.30
-	60.96	-
-	60.76	-
60.37	60.05	0.32
-	59.91	-
-	53.49	-
-	52.04	-
-	51.99	-
-	51.63	-
-	51.58	-
39.63	39.37	0.26
-	38.94	-
-	38.82	-
-	35.79	-
22.41	22.02	0.39

Table 4. 13 Comparison of ^{1}H NMR spectroscopic data of lacto-N-triose **(3)**

Reference[61] δ ^{1}H [ppm; mult; *J* (Hz)] 250 MHz	**Synthetic** δ ^{13}C (ppm) 600 MHz	**Deviation** (reference - synthetic) Δδ (ppm)
5.07; d; 3.6	5.23; d; 3.72	-0.16
4.56; d; 8.2	4.72; d; 8.58	-0.16
4.54; d; 8.0	4.71; d; 8.34	-0.17
-	4.68; d; 7.98	-
4.31; d; 7.8	4.45; d; 7.98	-0.14
-	4.1; d; 2.38	-
-	3.97-3.39; m	-
-	3.28; t; 8.4	-
1.90; s	2.07; s	-0.17

Table 4. 14 Comparison of ^{1}H NMR spectroscopic data of *epi*-Isoglobotriaose **(4)**

Reference[62] δ ^{1}H [ppm; *J* (Hz)] 250 MHz	**Synthetic** δ ^{13}C (ppm) 600 MHz	**Deviation** (reference - synthetic) Δδ (ppm)
5.224; 3.4	5.227; br	-0.003
4.667; 7.5	4.668; d; 7.44	-0.001
4.612; 8.0	4.617; d; 6.96	-0.005
4.511; 7.4	4.513; d; 7.02	-0.002
4.199; 2.8	4.297; br	-0.098
-	3.60-3.97; m	-
-	3.288; t; 7.74	-

References

1. Kilcoyne, M.; Joshi, L., Carbohydrates in therapeutics. *Cardiovasc Hematol Agents Med Chem* **2007,** *5*, 186-97.

2. Nicolaou, K. C.; Mitchell, H. J., Adventures in Carbohydrate Chemistry: New Synthetic Technologies, Chemical Synthesis, Molecular Design, and Chemical Biology. *Angew Chem Int Ed Engl* **2001,** *40*, 1576-1624.

3. Stallforth, P.; Lepenies, B.; Adibekian, A.; Seeberger, P. H., 2009 Claude S. Hudson Award in Carbohydrate Chemistry. Carbohydrates: a frontier in medicinal chemistry. *J Med Chem* **2009,** *52*, 5561-77.

4. Guo, J.; Ye, X. S., Protecting groups in carbohydrate chemistry: influence on stereoselectivity of glycosylations. *Molecules* **2010,** *15*, 7235-65.

5. Ranade, S.; Demchenko, A., Mechanism of Chemical Glycosylation: Focus on the Mode of Activation and Departure of Anomeric Leaving Groups. *Journal of Carbohydrate Chemistry* **2013,** *32*, 1-43.

6. Plante, O. J.; Palmacci, E. R.; Seeberger, P. H., Automated Synthesis of Polysaccharides. In *Methods in Enzymology*, Academic Press2003; Vol. 369, pp 235-248.

7. Crich, D., Mechanism of a chemical glycosylation reaction. *Acc Chem Res* **2010,** *43*, 1144-53.

8. Bohé, L.; Crich, D., A propos of glycosyl cations and the mechanism of chemical glycosylation; the current state of the art. *Carbohydr Res* **2015,** *403*, 48-59.

9. Boons, G. J.; Hale, K., *Organic Synthesis with Carbohydrates*. Sheffield Academic Press2000.

10. Schmidt, R.; Michel, J., Facile Sythesis of Alpha-O-Glycosyl and Beta-O-Glycosyl Imidates - Preparation of Glycosides and Disaccharides. *Angewandte Chemie-International Edition in English* **1980,** *19*, 731-732.

11. Ferrier, R.; Hay, R.; Vethaviyasar, N., Potentially Versatile Synthesis of Glycosides. *Carbohydrate Research* **1973,** *27*, 55-61.

12. Lian, G.; Zhang, X.; Yu, B., Thioglycosides in Carbohydrate Research. *Carbohydrate Research* **2015,** *403*, 13-22.

13. Fraser-Reid, B. O.; Tatsuta, K.; Thiem, J., *Glycoscience: Chemistry and Chemical Biology I–III*. Springer Berlin Heidelberg2012.

14. Teruaki, M.; Yoshiyuki, M.; Shin-ichiro, S., An Efficient Method for Glucosylation of Hydroxy Compounds Using Glucopyranosyl Fluoride. *Chemistry Letters* **1981,** *10*, 431-432.

15. Toshima, K., Glycosyl fluorides in glycosidations. *Carbohydr Res* **2000,** *327*, 15-26.

16. Kondo, H.; Ichikawa, Y.; Wong, C., Beta-Sialyl Phosphite and Phosphoramidite - Synthesis and Application to the Chemoenzymatic Synthesis of CMP-Sialic Acid and Sialyl Oligosaccharides. *Journal of the American Chemical Society* **1992,** *114*, 8748-8750.

17. Sim, M.; Kondo, H.; Wong, C., Synthesis and Use of Glycosyl Phosphites - An Effective Route to Glycosyl Phosphates, Sugar Nucleotides, and Glycosides. *Journal of the American Chemical Society* **1993,** *115*, 2260-2267.

18. Watanabe, Y.; Nakamoto, C.; Yamamoto, T.; Ozaki, S., Glycosylation Using Glycosyl Phosphite as a Glycosyl Donor. *Tetrahedron* **1994,** *50*, 6523-6536.

19. Boons, G.; Demchenko, A., Recent advances in O-sialylation. *Chemical Reviews* **2000,** *100*, 4539-+.

20. Kondo, H.; AokiI, S.; Ichikawa, Y.; Halcomb, R.; Ritzen, H.; Wong, C., Glycosyl Phopshites as Glycosylation Reagents - Scope and Mechanism. *Journal of Organic Chemistry* **1994,** *59*, 864-877.

21. Neubacher, B.; Schmidt, D.; Ziegelmuller, P.; Thiem, J., Preparation of sialylated oligosaccharides employing recombinant trans-sialidase from Trypanosoma cruzi. *Organic & Biomolecular Chemistry* **2005,** *3*, 1551-1556.

22. Yu, H.; Chokhawala, H.; Karpel, R.; Yu, H.; Wu, B.; Zhang, J.; Zhang, Y.; Jia, Q.; Chen, X., A Multifunctional Pasteurella multocida Sialyltransferase: A Powerful Tool for the Synthesis of Sialoside Libraries. *J Am Chem Soc* **2005,** *127*, 17618-17619.

23. Nahalka, J.; Patoprsty, V., Enzymatic synthesis of sialylation substrates powered by a novel polyphosphate kinase (PPK3). *Organic & Biomolecular Chemistry* **2009,** *7*, 1778-1780.

24. Schmolzer, K.; Czabany, T.; Luley-Goedl, C.; Pavkov-Keller, T.; Ribitsch, D.; Schwab, H.; Gruber, K.; Weber, H.; Nidetzky, B., Complete switch from alpha-2,3-to alpha-2,6-regioselectivity in Pasteurella dagmatis beta-D-galactoside sialyltransferase by active-site redesign. *Chem Commun* **2015,** *51*, 3083-3086.

25. Ogawa, T.; Sugimoto, M., Synthetic Studies on Cell-Surface Glycans .31. Synthesis of Alpha-Neu5acp-(2-]3)-D-Gal and Alpha-Neu5acp-(2-]3)-Beta-D-Galp-(1-]4)-D-Glc. *Carbohyd Res* **1985,** *135*, C5-C9.

26. Ito, Y.; Ogawa, T., Highly Stereoselective Glycosylation of Sialic-Acid Aided by Stereocontrolling Auxiliaries. *Tetrahedron* **1990,** *46*, 89-102.

27. Murata, T.; Morimoto, S.; Zeng, X. X.; Watanabe, S.; Usui, T., Enzymatic synthesis of alpha-L-fucosyl-N-acetyllactosamines and 3 '-O-alpha-L-fucosyllactose utilizing alpha-L-fucosidases. *Carbohyd Res* **1999,** *320*, 192-199.

28. Fang, J. W.; Li, J.; Chen, X.; Zhang, Y. N.; Wang, J. Q.; Guo, Z. M.; Zhang, W.; Yu, L. B.; Brew, K.; Wang, P. G., Highly efficient chemoenzymatic synthesis of alpha-galactosyl

epitopes with a recombinant alpha(1 -> 3)-galactosyltransferase. *J Am Chem Soc* **1998,** *120*, 6635-6638.

29. Zhou, G. Y.; Liu, X. W.; Su, D. R.; Li, L.; Xiao, M.; Wang, P. G., Large scale enzymatic synthesis of oligosaccharides and a novel purification process. *Bioorg Med Chem Lett* **2011,** *21*, 311-314.

30. Yamada, A.; Hatano, K.; Koyama, T.; Matsuoka, K.; Takahashi, N.; Hidari, K. I. P. J.; Suzuki, T.; Suzuki, Y.; Terunuma, D., Lactotriaose-containing carbosilane dendrimers: Syntheses and lectin-binding activities. *Bioorgan Med Chem* **2007,** *15*, 1606-1614.

31. Allen, J. R.; Harris, C. R.; Danishefsky, S. J., Pursuit of optimal carbohydrate-based anticancer vaccines: preparation of a multiantigenic unimolecular glycopeptide containing the Tn, MBr1, and Lewis(y) antigens. *J Am Chem Soc* **2001,** *123*, 1890-7.

32. Matsuoka, K.; Goshu, Y.; Takezawa, Y.; Mori, T.; Sakamoto, J.; Yamada, A.; Onaga, T.; Koyama, T.; Hatano, K.; Snyder, P.; Toone, E.; Terunuma, D., Practical synthesis of fully protected globotriaose and its glycopolymers. *Carbohyd Polym* **2006,** *69*, 326-335.

33. Koike, K.; Sugimoto, M.; Sato, S.; Ito, Y.; Nakahara, Y.; Ogawa, T., Total synthesis of globotriaosyl-E and Z-ceramides and isoglobotriaosyl-E-ceramide. *Carbohydr Res* **1987,** *163*, 189-208.

34. Allen, J.; Danishefsky, S., New applications of the n-pentenyl glycoside method in the synthesis and immunoconjugation of fucosyl GM(1): A highly tumor-specific antigen associated with small cell lung carcinoma. *Journal of the American Chemical Society* **1999,** *121*, 10875-10882.

35. Greenwald, R. B., PEG drugs: an overview. *J Control Release* **2001,** *74*, 159-71.

36. Nicolaou, K.; Caufield, T.; Katoaoka, H.; Stylianides, N., Total Synthesis of the Tumor-Associated Le(X) Family of Glycosphingolipids. *Journal of the American Chemical Society* **1990,** *112*, 3693-3695.

37. Nagorny, P.; Fasching, B.; Li, X.; Chen, G.; Aussedat, B.; Danishefsky, S., Toward Fully Synthetic Homogeneous beta-Human Follicle-Stimulating Hormone (beta-hFSH) with a Biantennary N-Linked Dodecasaccharide. Synthesis of beta-hFSH with Chitobiose Units at the Natural Linkage Sites. *Journal of the American Chemical Society* **2009,** *131*, 5792-5799.

38. Malapelle, A.; Coslovi, A.; Doisneau, G.; Beau, J., An expeditious synthesis of N-acetylneuraminic acid alpha-C-glycosyl derivatives ("alpha-C-glycosides") from the anomeric acetates. *European Journal of Organic Chemistry* **2007**, 3145-3157.

39. Cao, S.; Meunier, S.; Andersson, F.; Letellier, M.; Roy, R., Mild Stereoselective Syntheses of Thioglycosides under PTC Conditions and their Use as Active and Latent Glycosyl Donors. *Tetrahedron-Asymmetry* **1994,** *5*, 2303-2312.

40. Dullenkopf, W.; Castro-Palomino, J. C.; Manzoni, L.; Schmidt, R. R., N-trichloroethoxycarbonyl-glucosamine derivatives as glycosyl donors. *Carbohydr Res* **1996,** *296*, 135-47.

41. Allman, S. A.; Jensen, H. H.; Vijayakrishnan, B.; Garnett, J. A.; Leon, E.; Liu, Y.; Anthony, D. C.; Sibson, N. R.; Feizi, T.; Matthews, S.; Davis, B. G., Potent fluoro-oligosaccharide probes of adhesion in Toxoplasmosis. *Chembiochem* **2009,** *10*, 2522-9.

42. Kitowski, A.; Jimenez-Moreno, E.; Salvado, M.; Mestre, J.; Castillon, S.; Jimenez-Oses, G.; Boutureira, O.; Bemardes, G., Oxidative Activation of C-S Bonds with an Electropositive Nitrogen Promoter Enables Orthogonal Glycosylation of Alkyl over Phenyl Thioglycosides. *Organic Letters* **2017,** *19*, 5490-5493.

43. Dinkelaar, J.; de Jong, A. R.; van Meer, R.; Somers, M.; Lodder, G.; Overkleeft, H. S.; Codee, J. D.; van der Marel, G. A., Stereodirecting effect of the pyranosyl C-5 substituent in glycosylation reactions. *J Org Chem* **2009,** *74*, 4982-91.

44. Nicolaou, K. C.; Caulfield, T. J.; Kataoka, H.; Stylianides, N. A., Total Synthesis of the Tumor-Associated Le(X) Family of Glycosphingolipids. *J Am Chem Soc* **1990,** *112*, 3693-3695.

45. Chervin, S. M.; Lowe, J. B.; Koreeda, M., Synthesis and biological evaluation of a new sialyl Lewis X mimetic derived from lactose. *J Org Chem* **2002,** *67*, 5654-5662.

46. Boons, G. J.; Demchenko, A. V., Recent advances in O-sialylation. *Chemical Reviews* **2000,** *100*, 4539-+.

47. Kondo, H.; Ichikawa, Y.; Wong, C. H., Beta-Sialyl Phosphite and Phosphoramidite - Synthesis and Application to the Chemoenzymatic Synthesis of Cmp-Sialic Acid and Sialyl Oligosaccharides. *J Am Chem Soc* **1992,** *114*, 8748-8750.

48. Nicolaou, K. C.; Bockovich, N. J.; Carcanague, D. R., Total synthesis of sulfated Lex and Lea-type oligosaccharide selectin ligands. *Journal of the American Chemical Society* **1993,** *115*, 8843-8844.

49. Lay, L.; Manzoni, L.; Schmidt, R. R., Synthesis of N-acetylglucosamine containing Lewis A and Lewis X building blocks based on N-tetrachlorophthaloyl protection--synthesis of Lewis X pentasaccharide. *Carbohydr Res* **1998,** *310*, 157-71.

50. Horton, D., *Advances in Carbohydrate Chemistry and Biochemistry*. Elsevier Science1998.

51. Zhou, Y.; Li, J.; Zhan, Y.; Pei, Z.; Dong, H., Halide promoted organotin-mediated carbohydrate benzylation: mechanism and application. *Tetrahedron* **2013,** *69*, 2693-2700.

52. Lawandi, J.; Rocheleau, S.; Moitessier, N., Regioselective acylation, alkylation, silylation and glycosylation of monosaccharides. *Tetrahedron* **2016,** *72*, 6283-6319.

53. Kong, X.; Grindley, T. B., Control of regioselectivity in reactions of dialkylstannylene acetals. Part II. NMR results and mechanistic analysis. *Canadian Journal of Chemistry* **1994,** *72*, 2405-2415.

54. David, S.; Hanessian, S., Regioselective manipulation of hydroxyl groups via organotin derivatives. *Tetrahedron* **1985,** *41*, 643-663.

55. Bode, L., Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* **2012,** *22*, 1147-62.

56. Ackerman, D. L.; Craft, K. M.; Townsend, S. D., Infant food applications of complex carbohydrates: Structure, synthesis, and function. *Carbohydr Res* **2017,** *437*, 16-27.

57. Sitkiewicz, I.; Green, N. M.; Guo, N.; Bongiovanni, A. M.; Witkin, S. S.; Musser, J. M., Transcriptome adaptation of group B Streptococcus to growth in human amniotic fluid. *PLoS One* **2009,** *4*, e6114.

58. Mereghetti, L.; Sitkiewicz, I.; Green, N. M.; Musser, J. M., Extensive adaptive changes occur in the transcriptome of Streptococcus agalactiae (group B streptococcus) in response to incubation with human blood. *PLoS One* **2008,** *3*, e3143.

59. Murata, T.; Morimoto, S.; Zeng, X.; Watanabe, S.; Usui, T., Enzymatic synthesis of alpha-L-fucosyl-N-acetyllactosamines and 3'-O-alpha-L-fucosyllactose utilizing alpha-L-fucosidases. *Carbohydr Res* **1999,** *320*, 192-9.

60. Liu, Y.; Feizi, T.; Campanero-Rhodes, M. A.; Childs, R. A.; Zhang, Y.; Mulloy, B.; Evans, P. G.; Osborn, H. M.; Otto, D.; Crocker, P. R.; Chai, W., Neoglycolipid probes prepared via oxime ligation for microarray analysis of oligosaccharide-protein interactions. *Chem Biol* **2007,** *14*, 847-59.

61. Paulsen, H.; Hadamczyk, D.; Kutschker, W.; Bunsch, A., Building Units of Oligosaccharides .58. Regioselective Glycosylation at 3'-Oh or 4'-Oh of Lactose Using Different Catalyst Systems. *Liebigs Annalen Der Chemie* **1985**, 129-141.

62. Urashima, T.; Sun, Y.; Fukuda, K.; Hirayama, K.; Taufik, E.; Nakamura, T.; Saito, T.; Merchant, J.; Green, B.; Messer, M., Chemical characterization of milk oligosaccharides of the eastern quoll (Dasyurus viverrinus). *Glycoconj J* **2015,** *32*, 361-70.

CHAPTER IV

Appendix A3:

Figures and Supplemental Data Relevant to Chapter IV

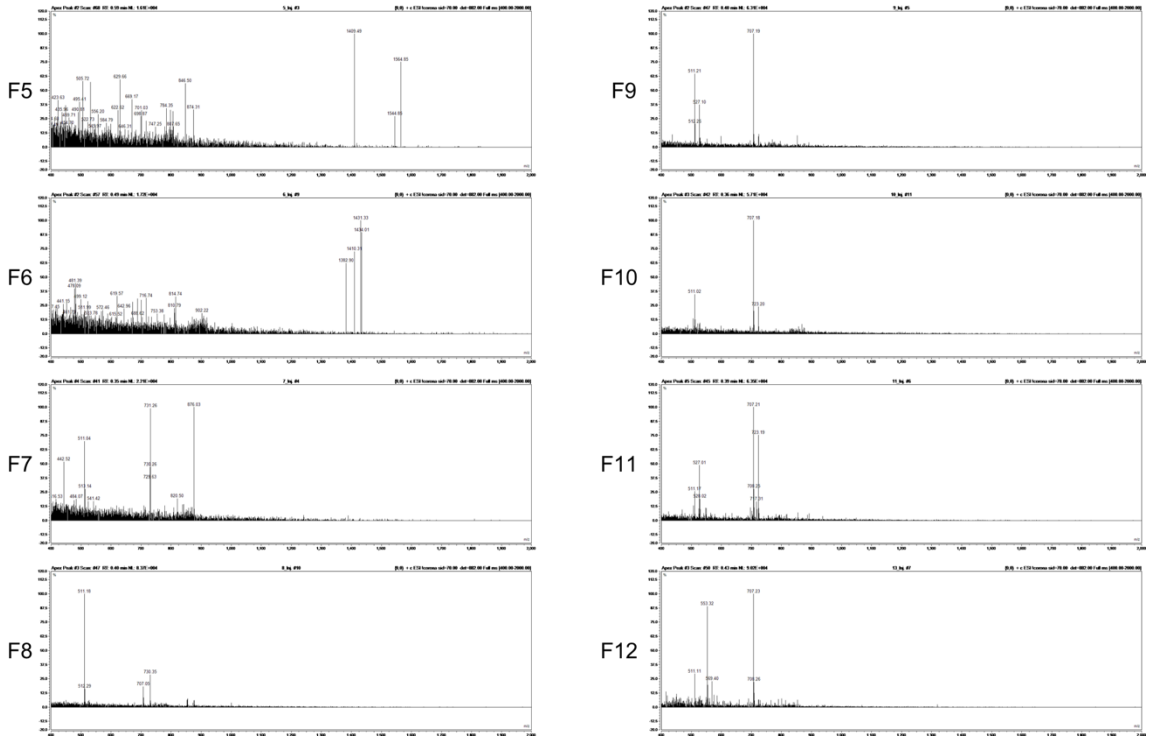


Figure A3. 1 Fractionation of HMOs from Donor 42. Fractions were characterized by LCMS.

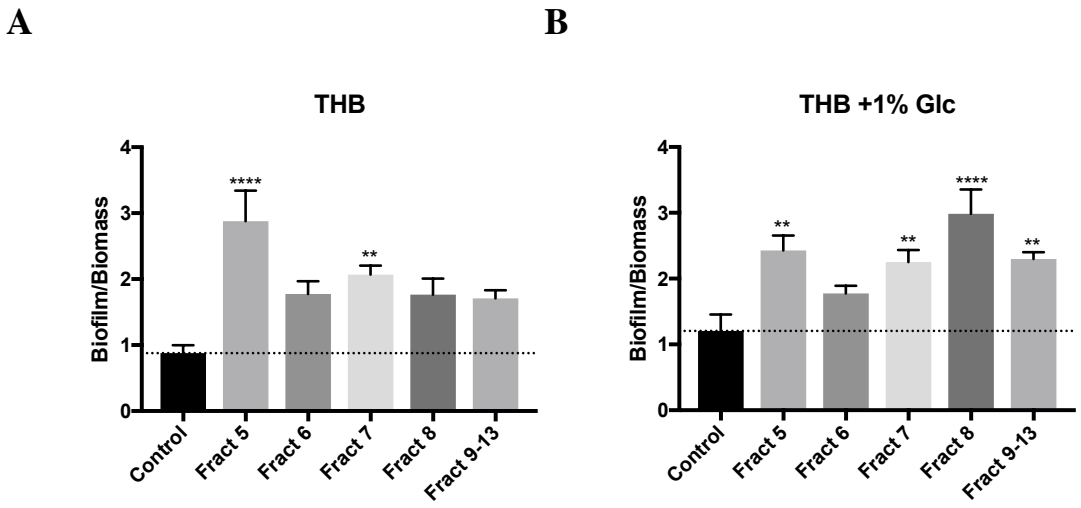


Figure A3. 2 Biofilm to biomass ratio for *S. agalactiae* strain CNCTC 10/84 after 24 h of growth in media alone or in the presence of ca. 5 mg/mL fractionated HMOs from Donor 42 (A) THB medium alone. Data represented as the mean biofilm/biomass ratio ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA, $F = 6.969$ with posthoc Dunnett's multiple comparison test comparing each HMO sample against the control sample without HMOs. (B) THB medium supplemented with 1% glucose. Data are expressed as the mean biofilm/biomass ratio ± SEM of 3 separate experiments, each with 3 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by oneway ANOVA, $F = 7.233$ with posthoc Dunnet's multiple comparison, compared to media alone.

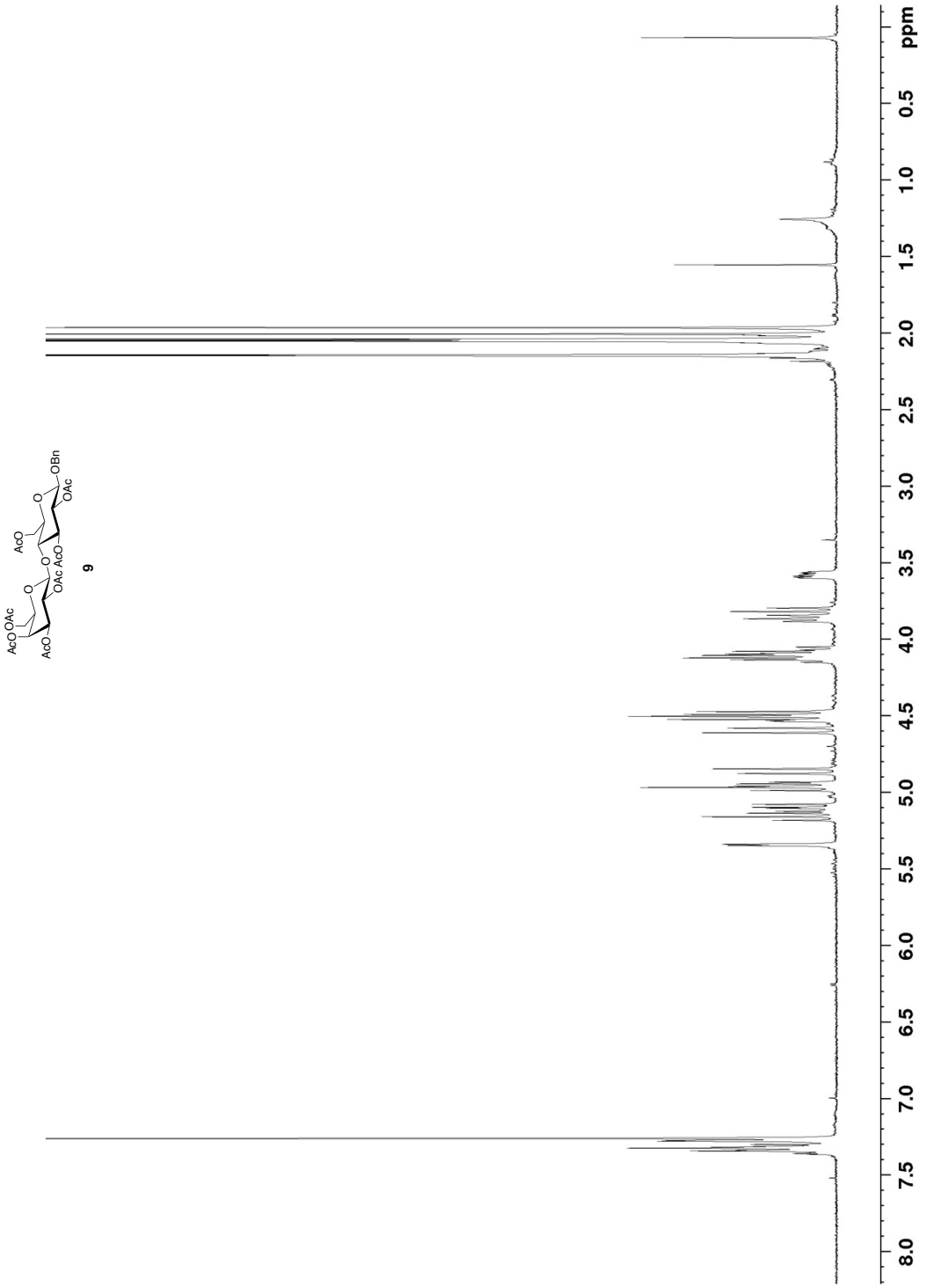


Figure A3. 3 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 9

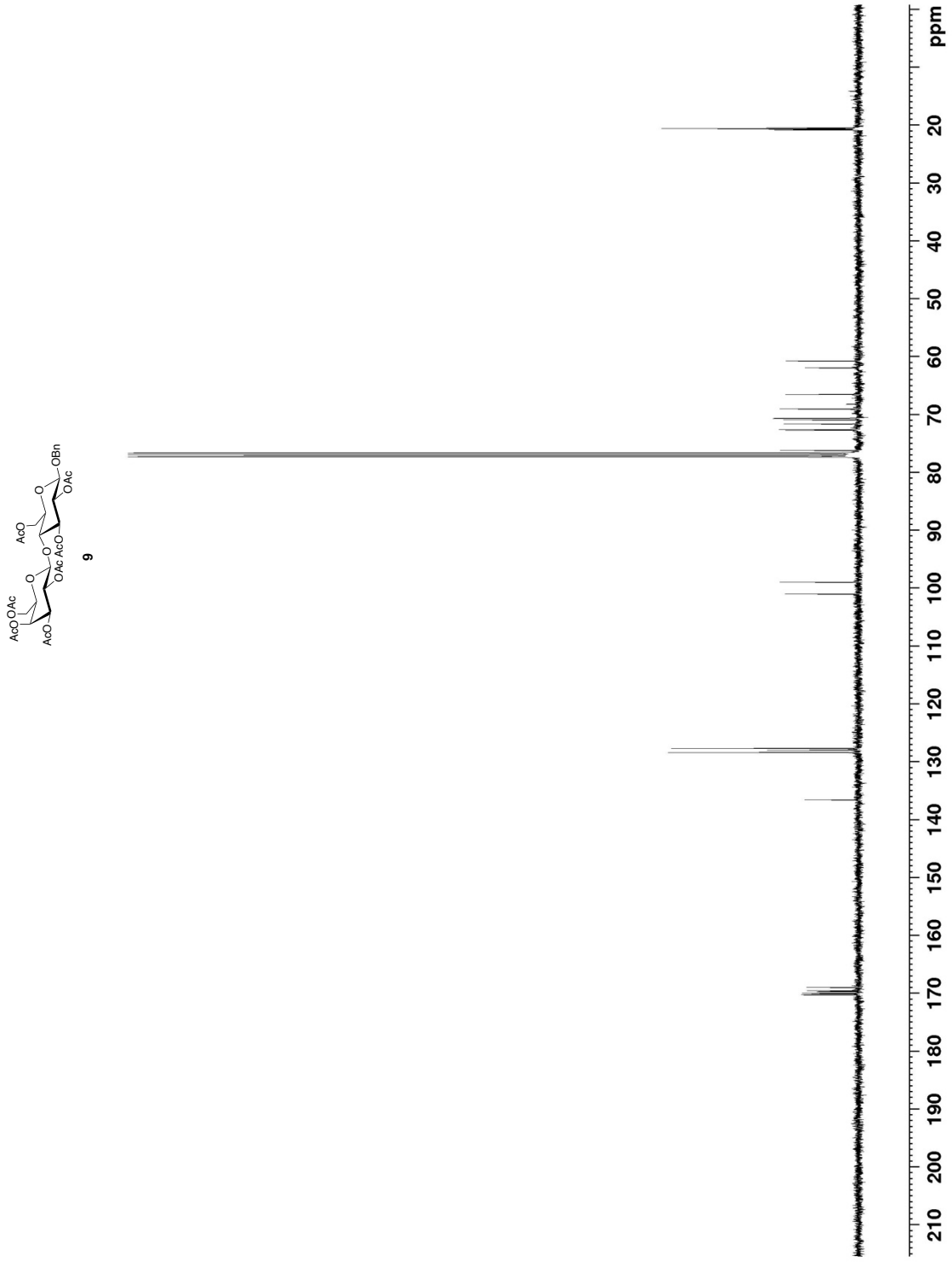


Figure A3. 4 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 9

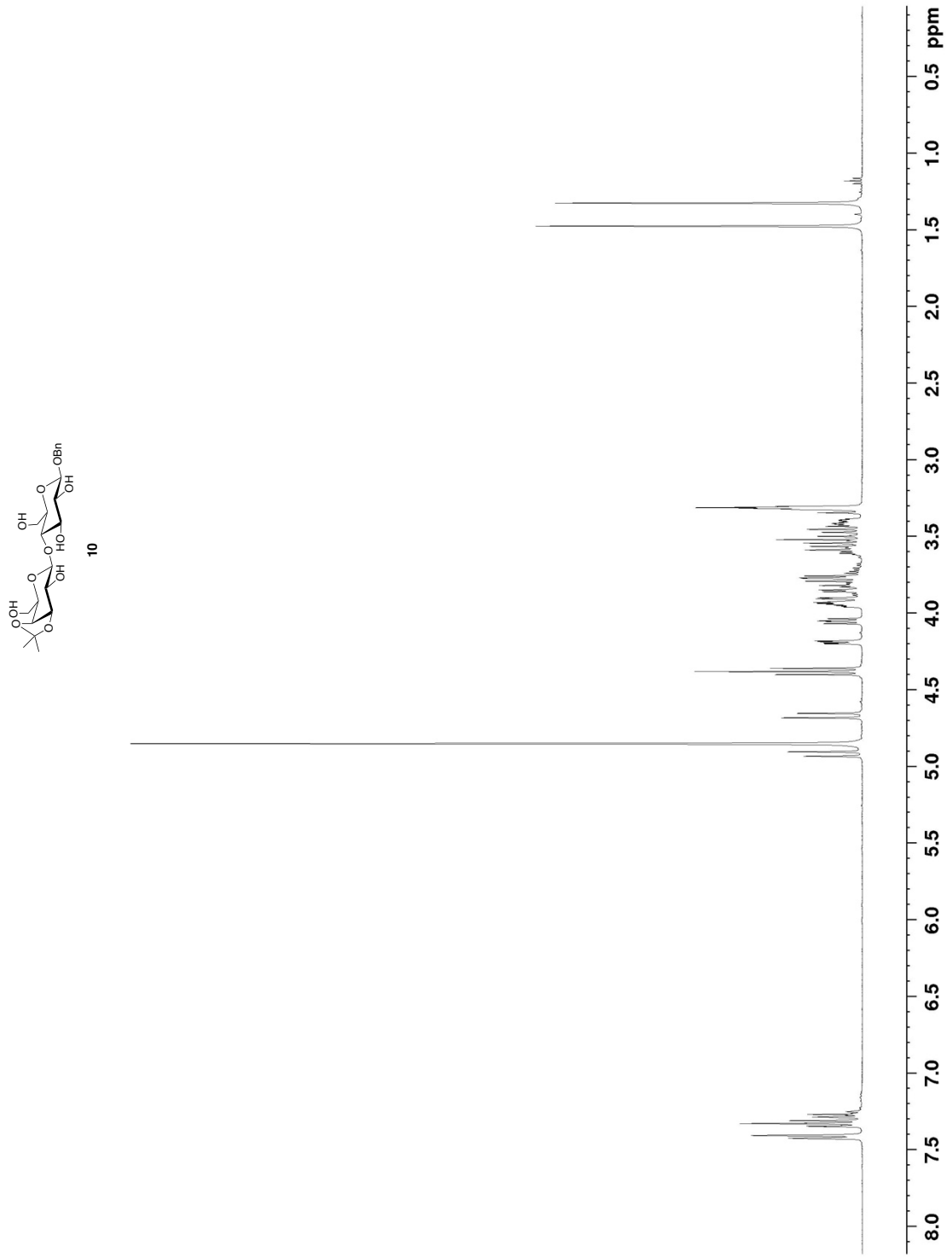


Figure A3. 5 ^{1}H NMR spectra (400 MHz, MeOD) of compound 10

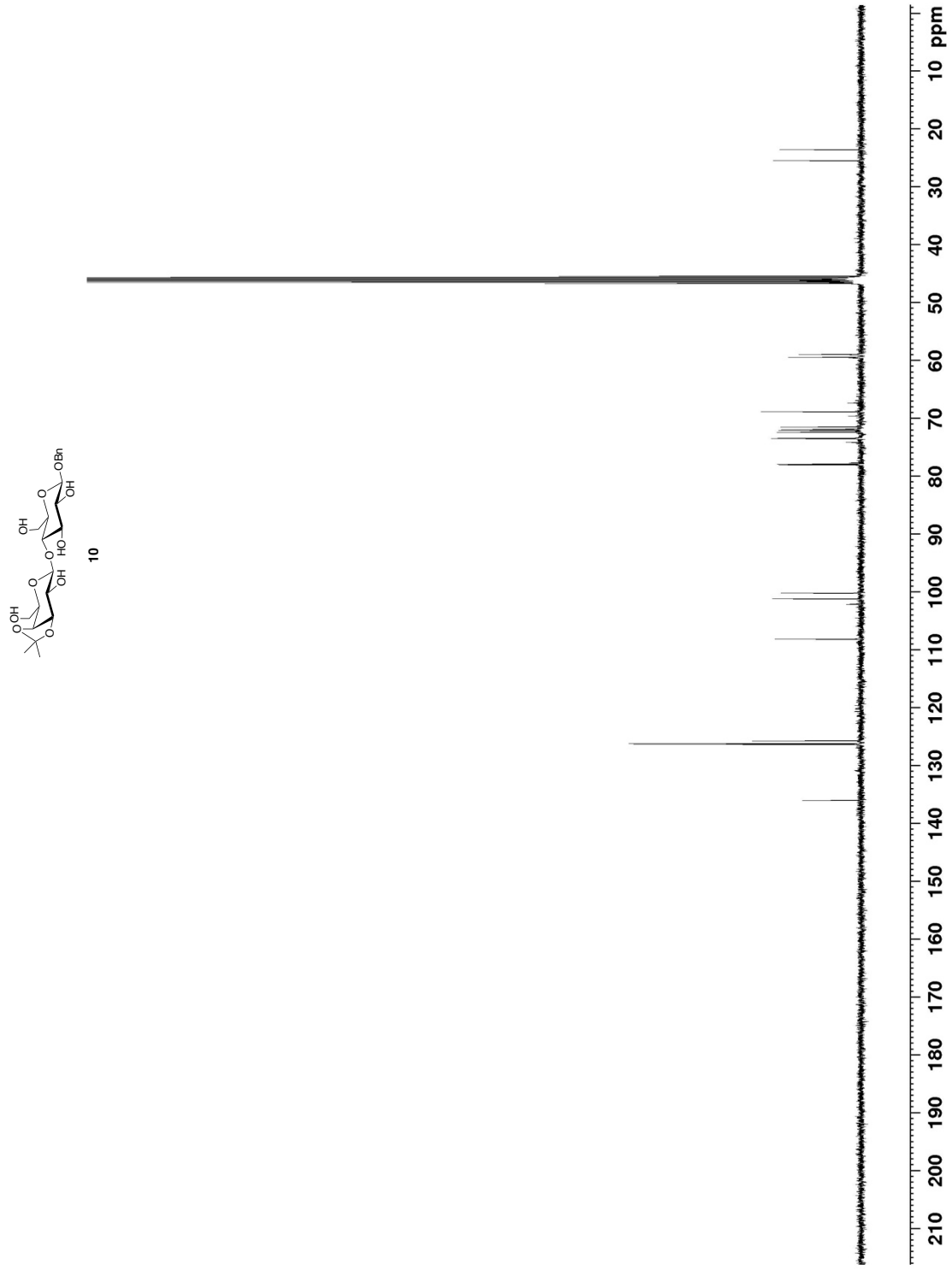


Figure A3. 6 ^{13}C NMR spectra (100 MHz, MeOD) of compound 10

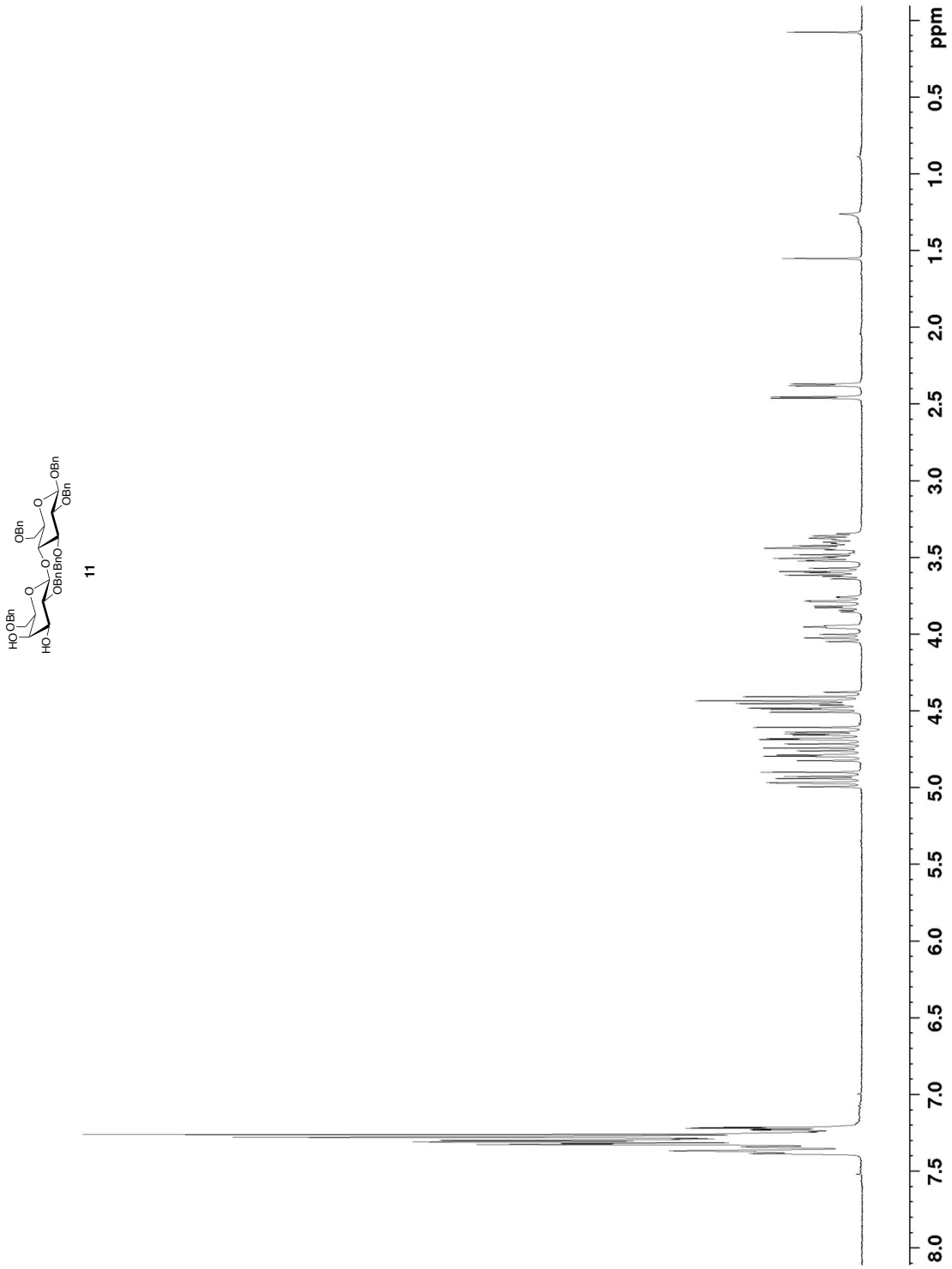


Figure A3. 7 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 11

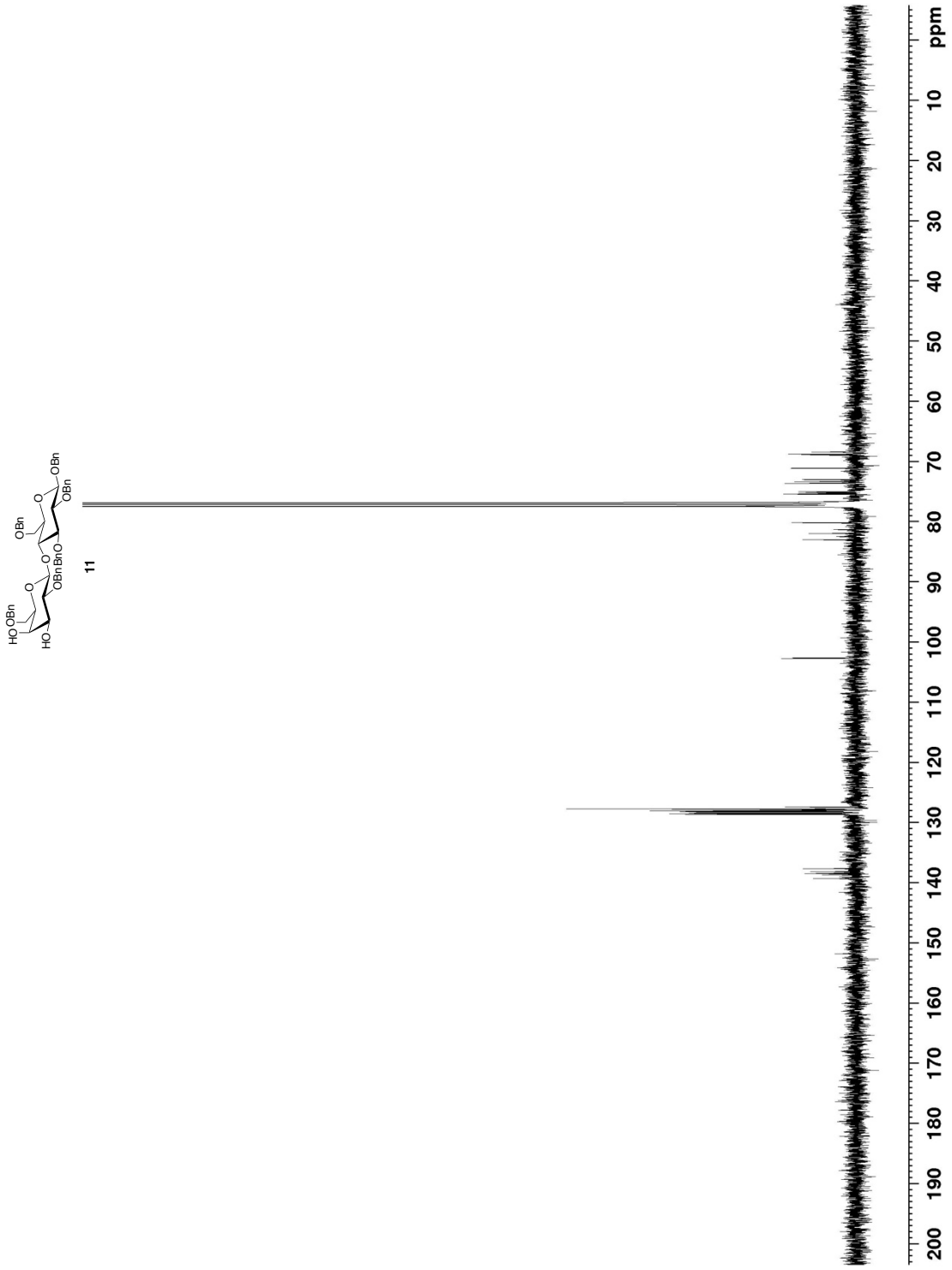


Figure A3. 8 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 11

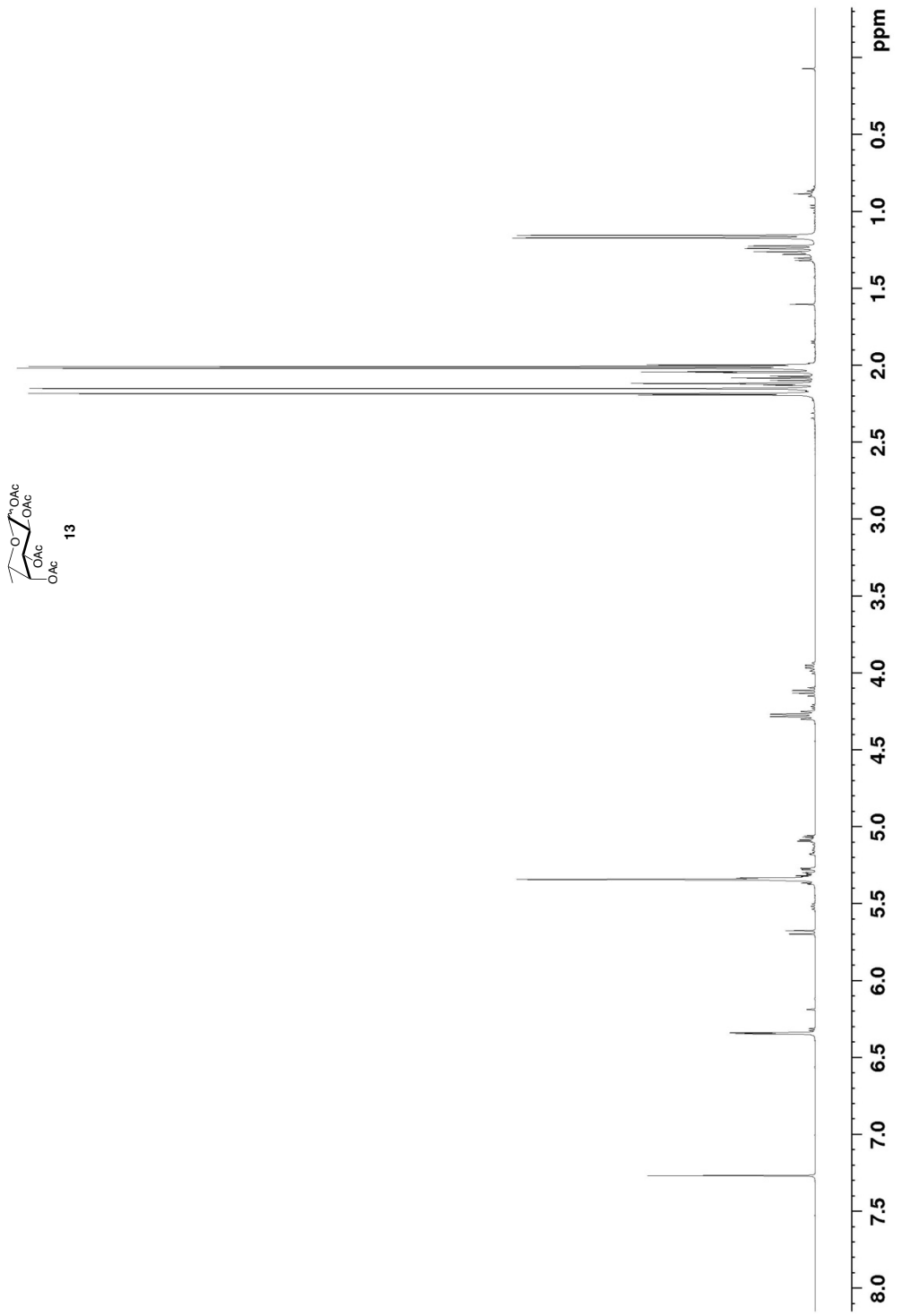


Figure A3. 9 ^{1}H NMR spectra (400 MHz, CDCl3) of compound 13

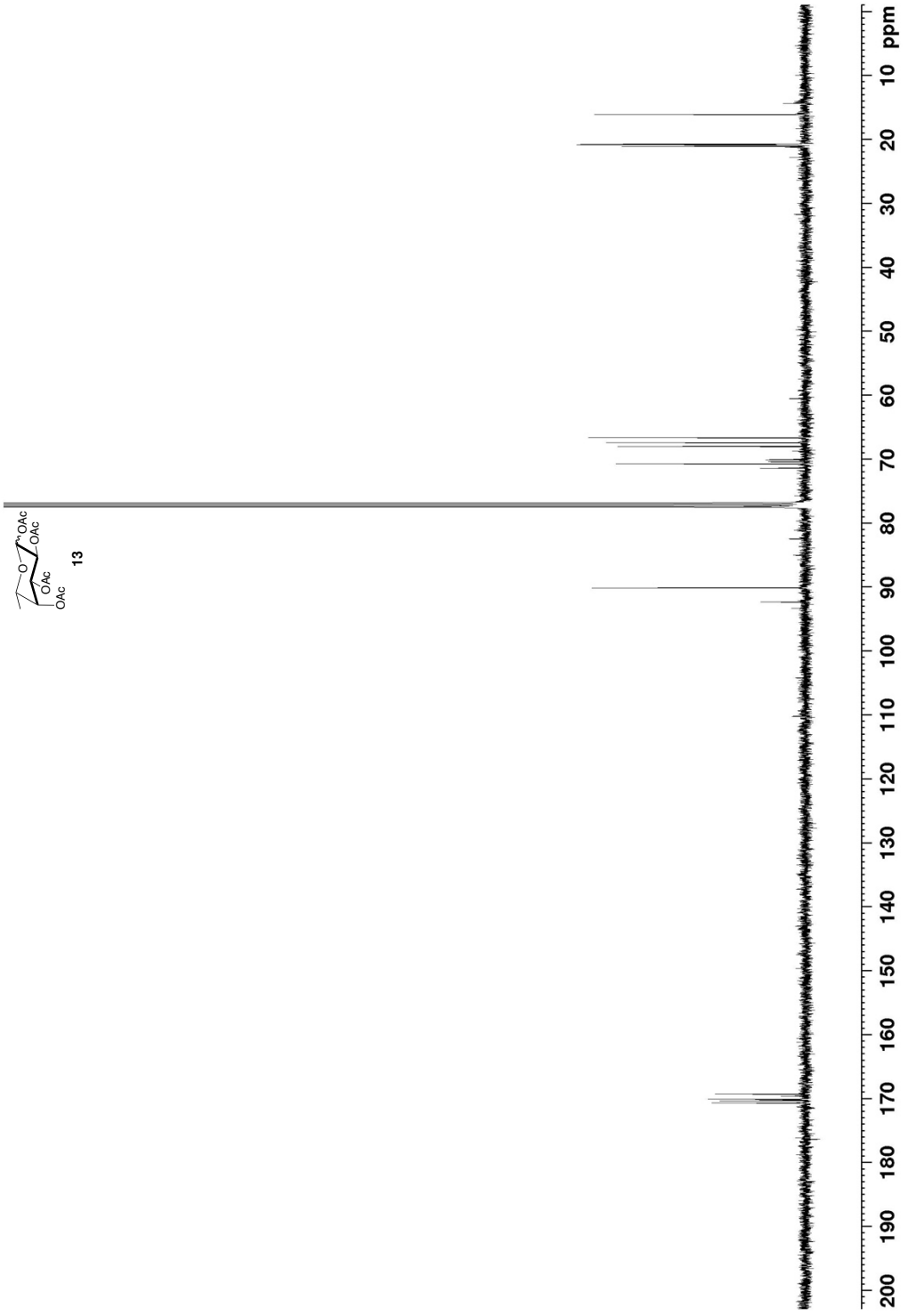


Figure A3. 10 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 13

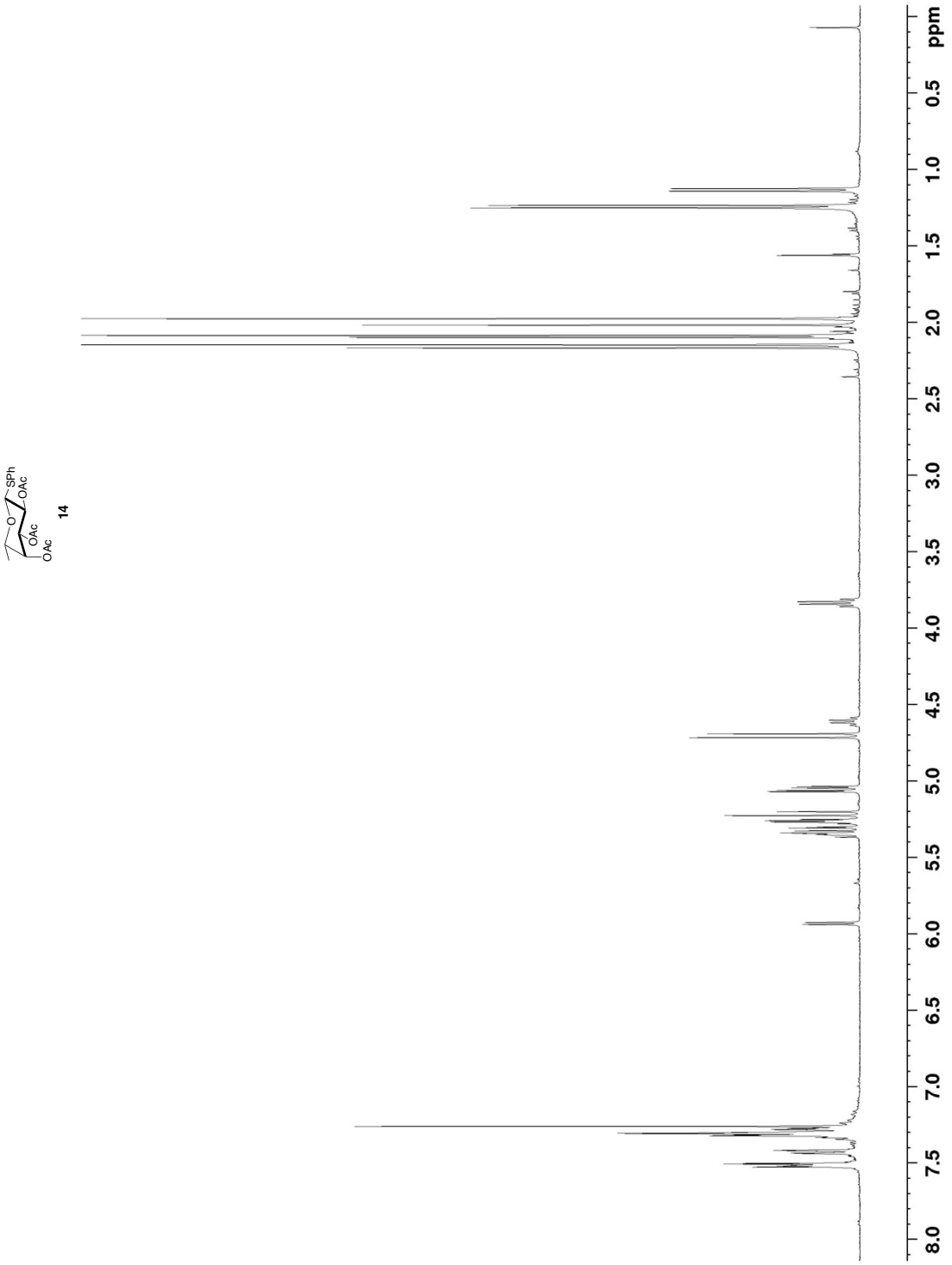


Figure A3. 11 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 14

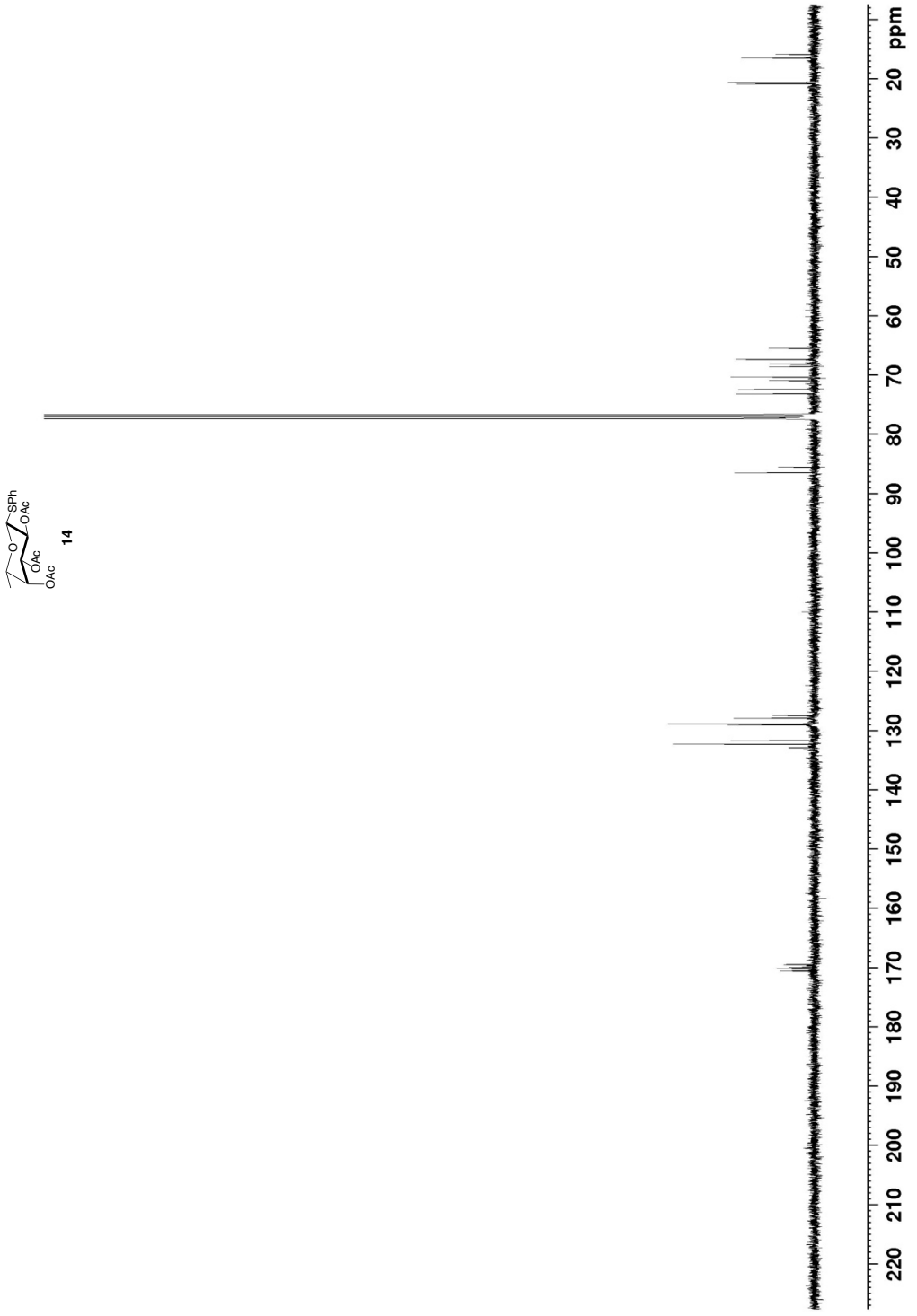


Figure A3. 12 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 14

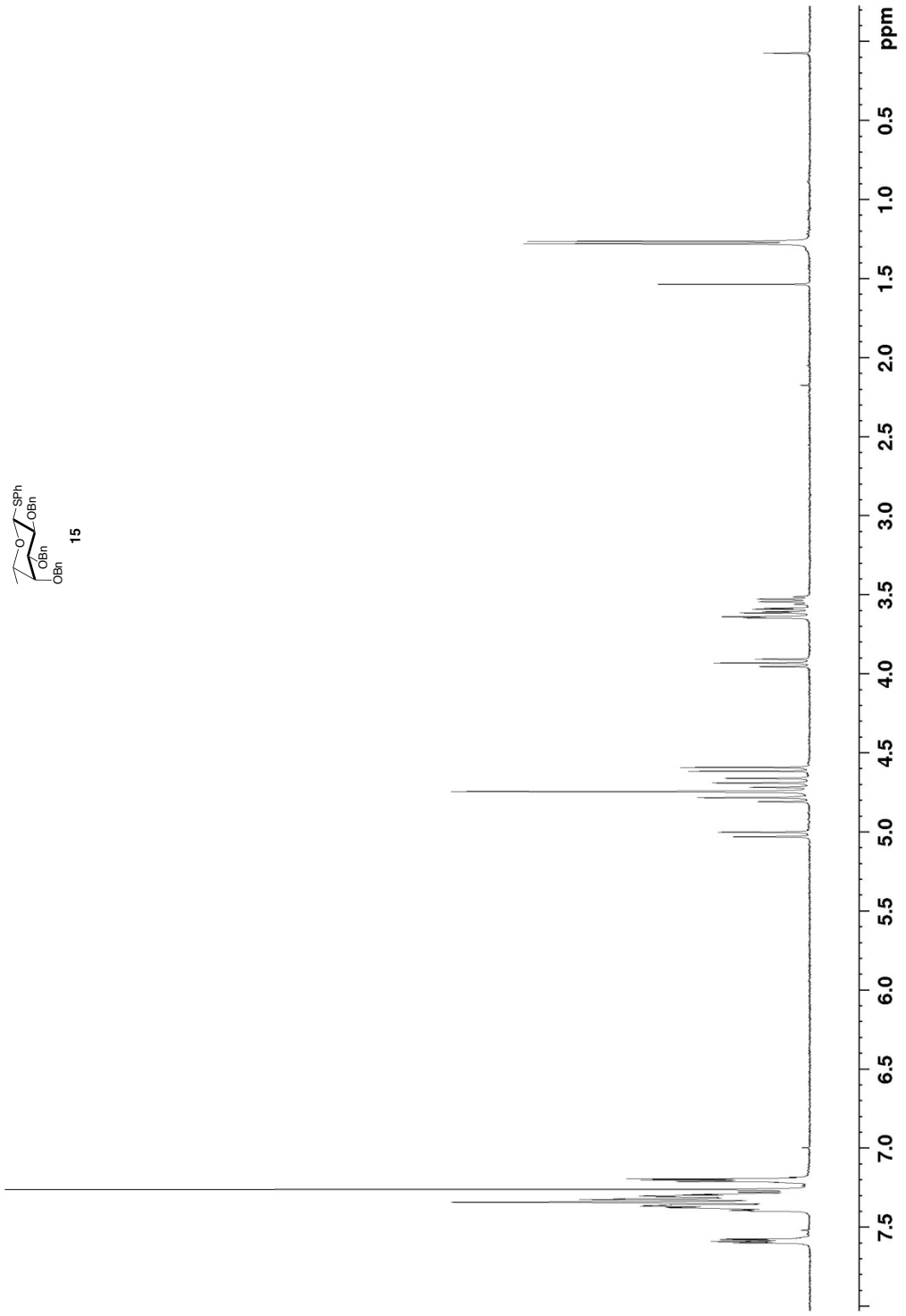


Figure A3. 13 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 15

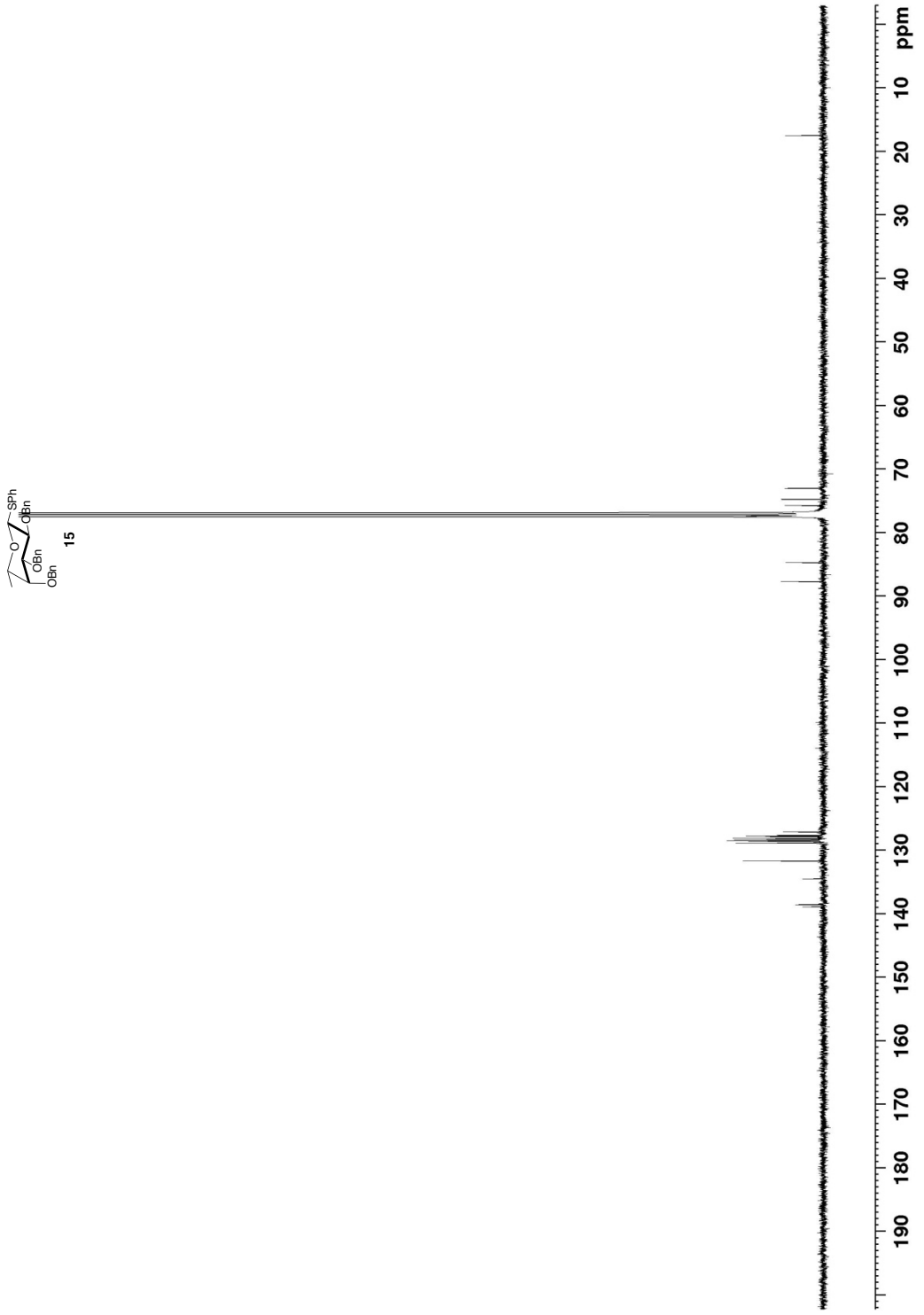


Figure A3. 14 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 15

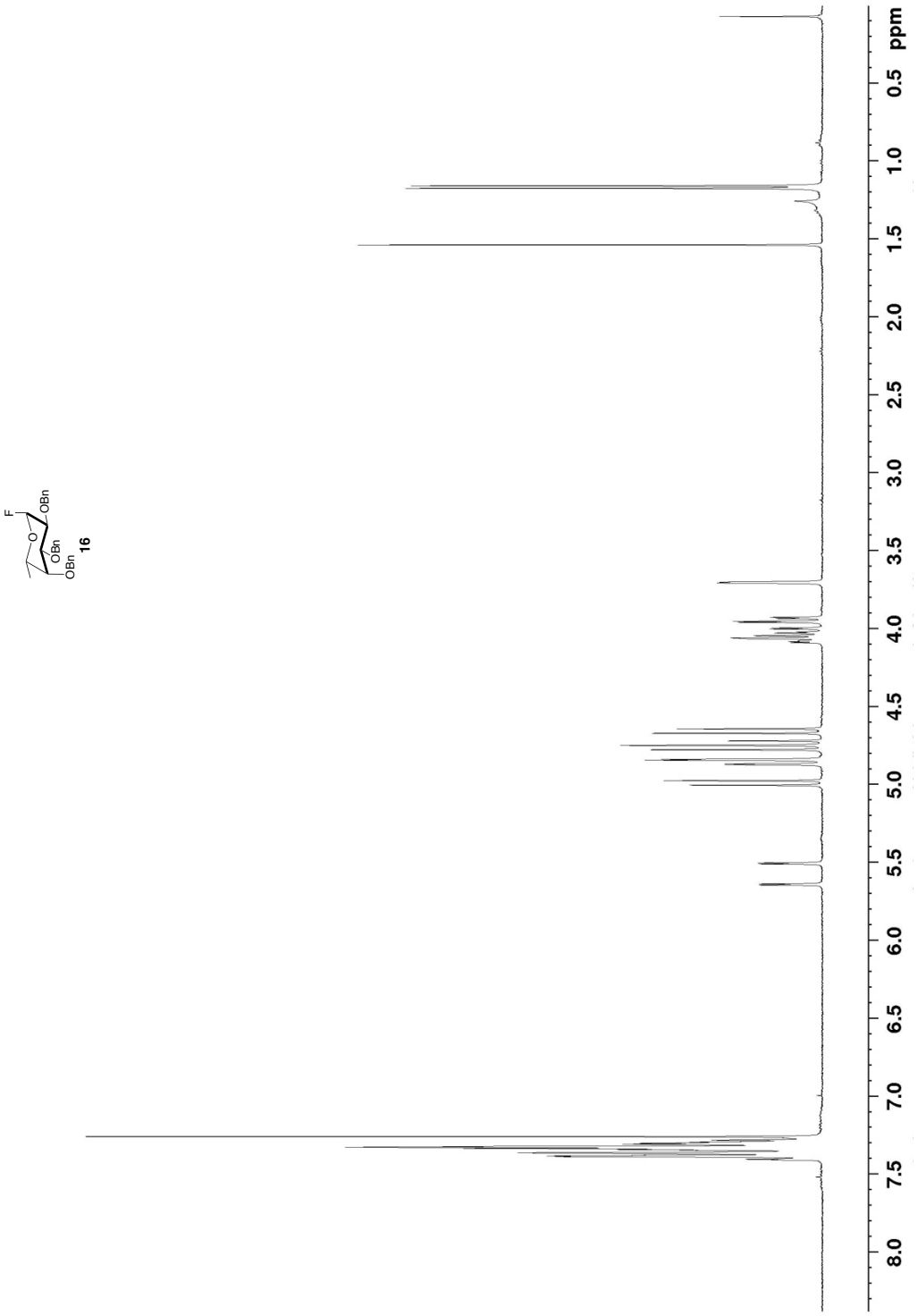


Figure A3. 15 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 16

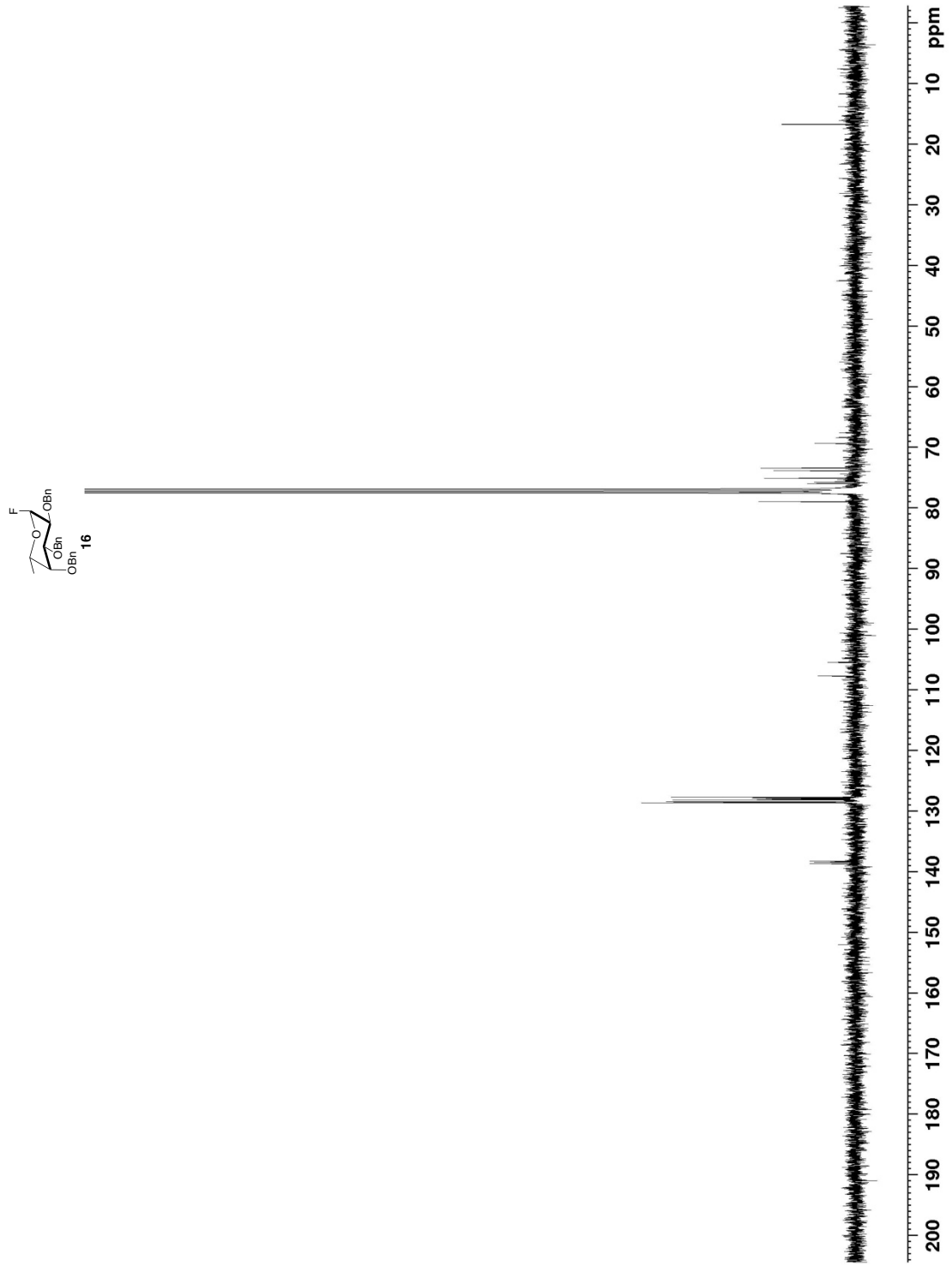


Figure A3. 16 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 16

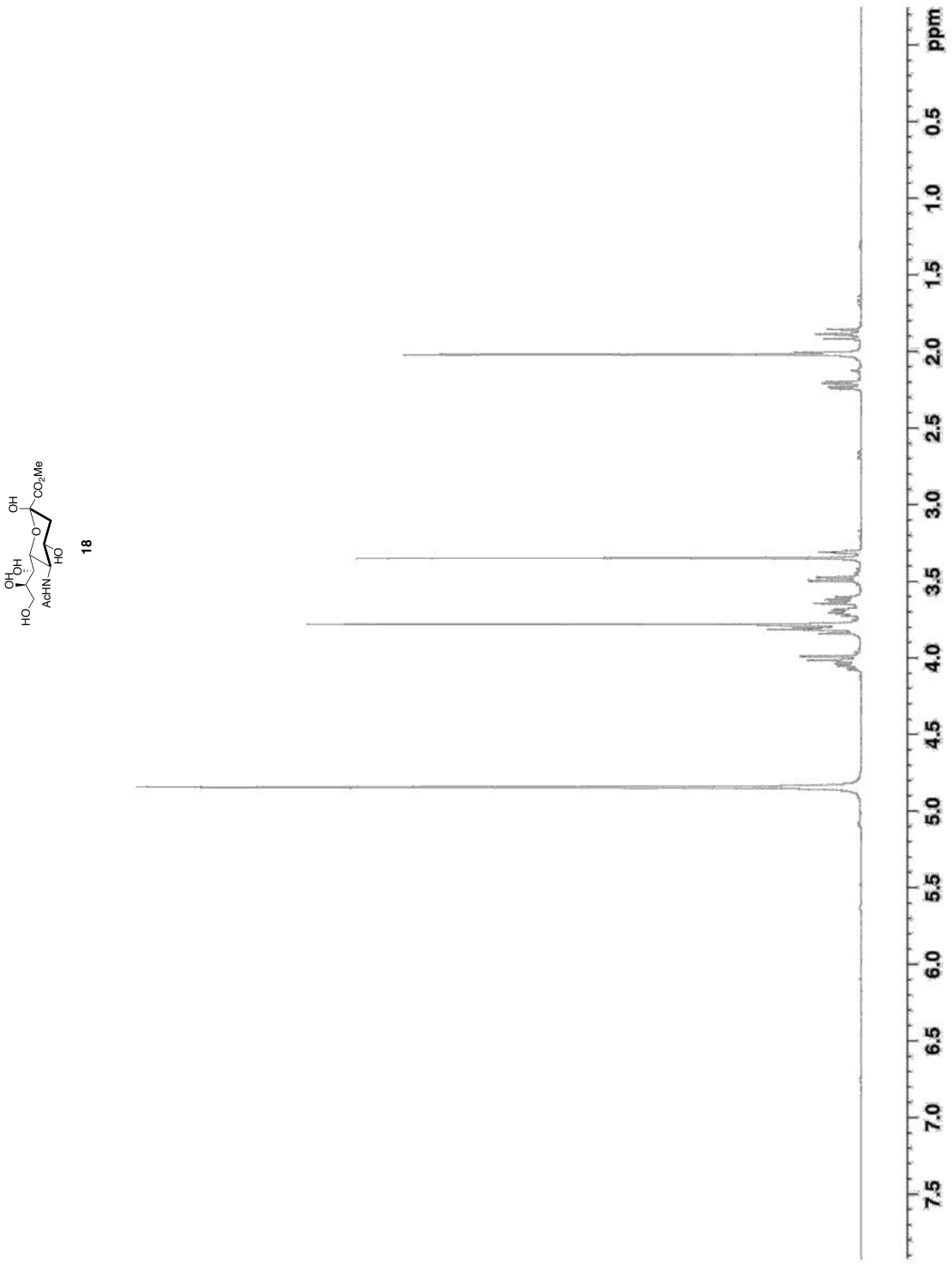


Figure A3. 17 ^{1}H NMR spectra (400 MHz, MeOD) of compound 18

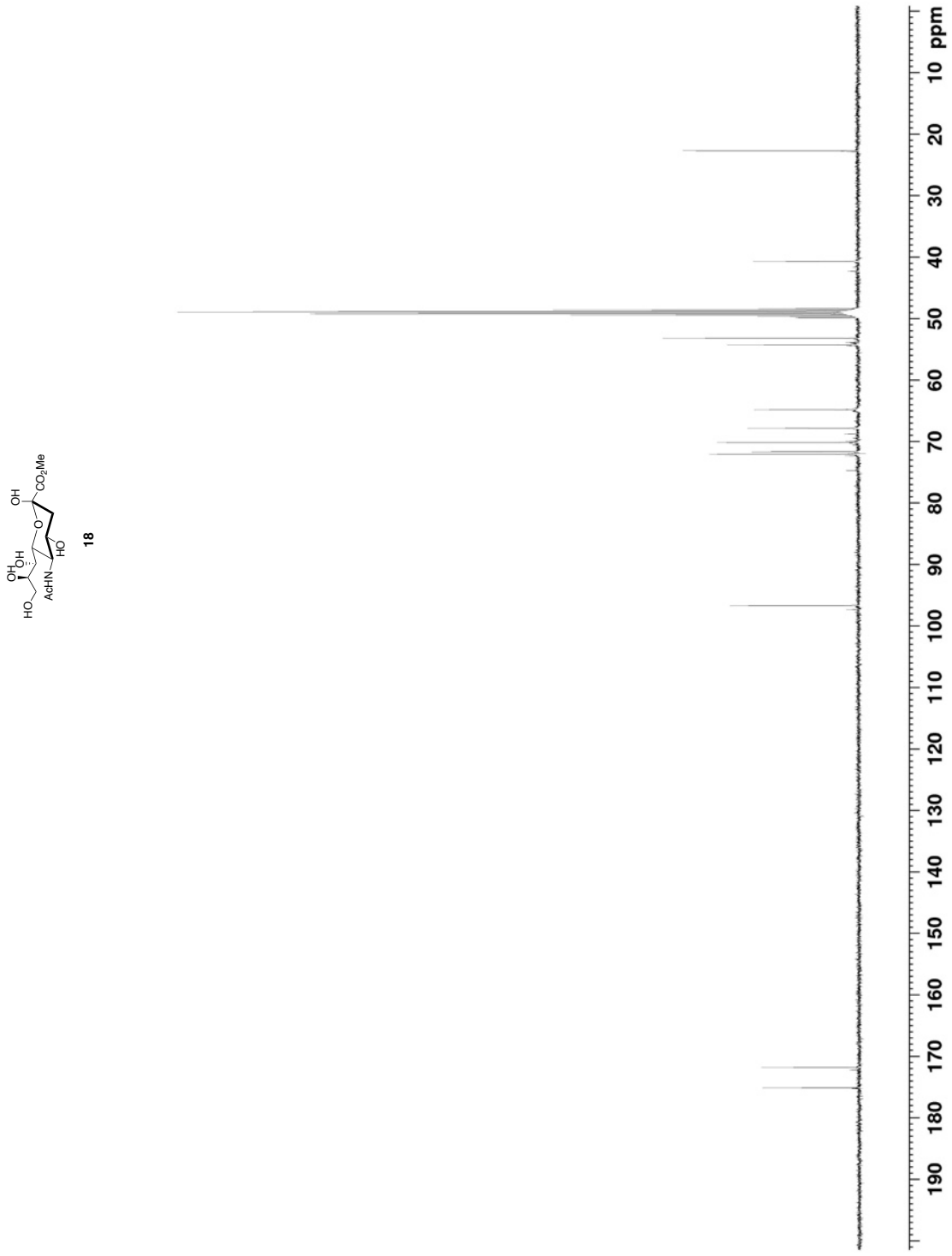


Figure A3. 18 ^{13}C NMR spectra (100 MHz, MeOD) of compound 18

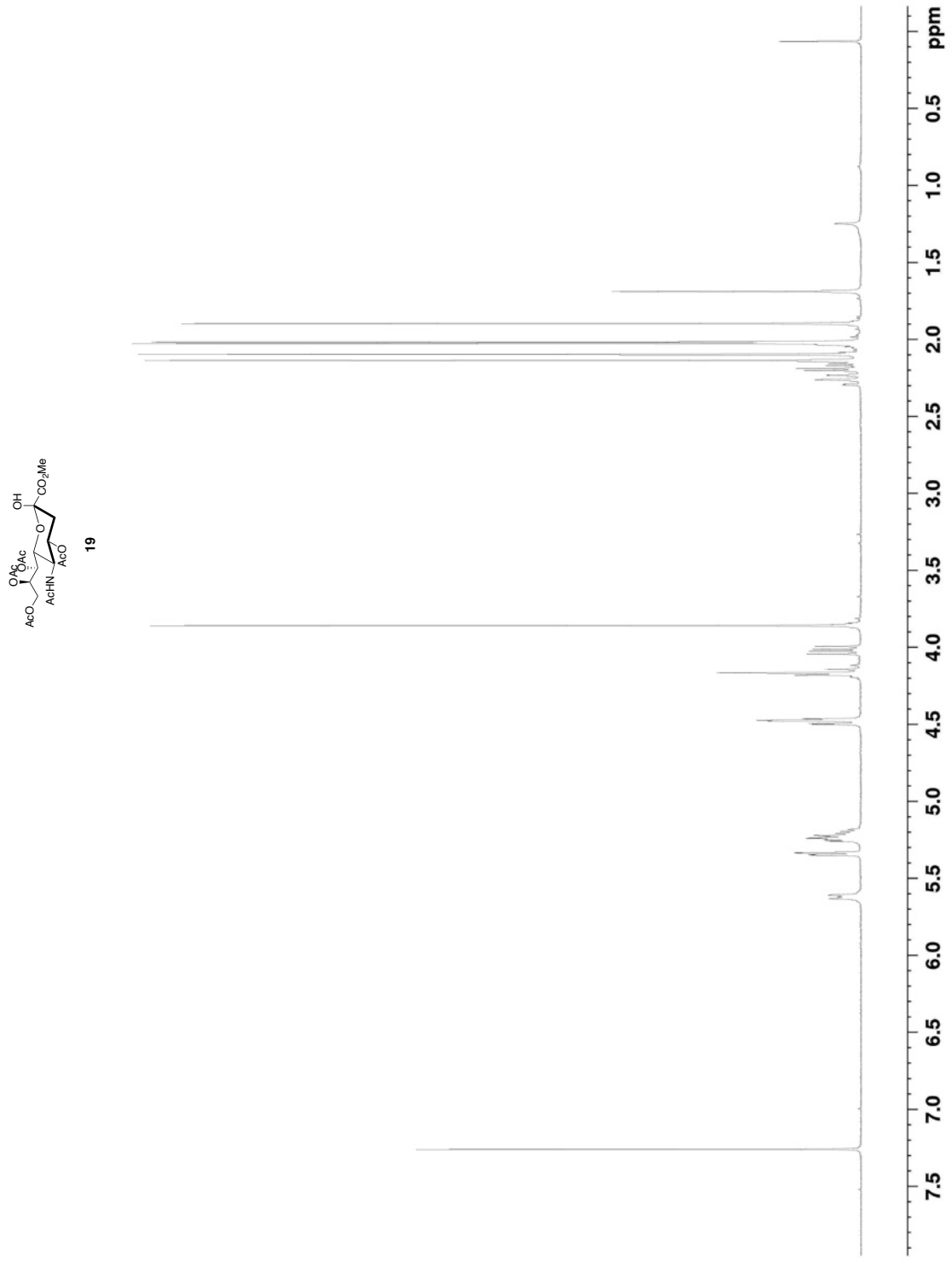


Figure A3. 19 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 19

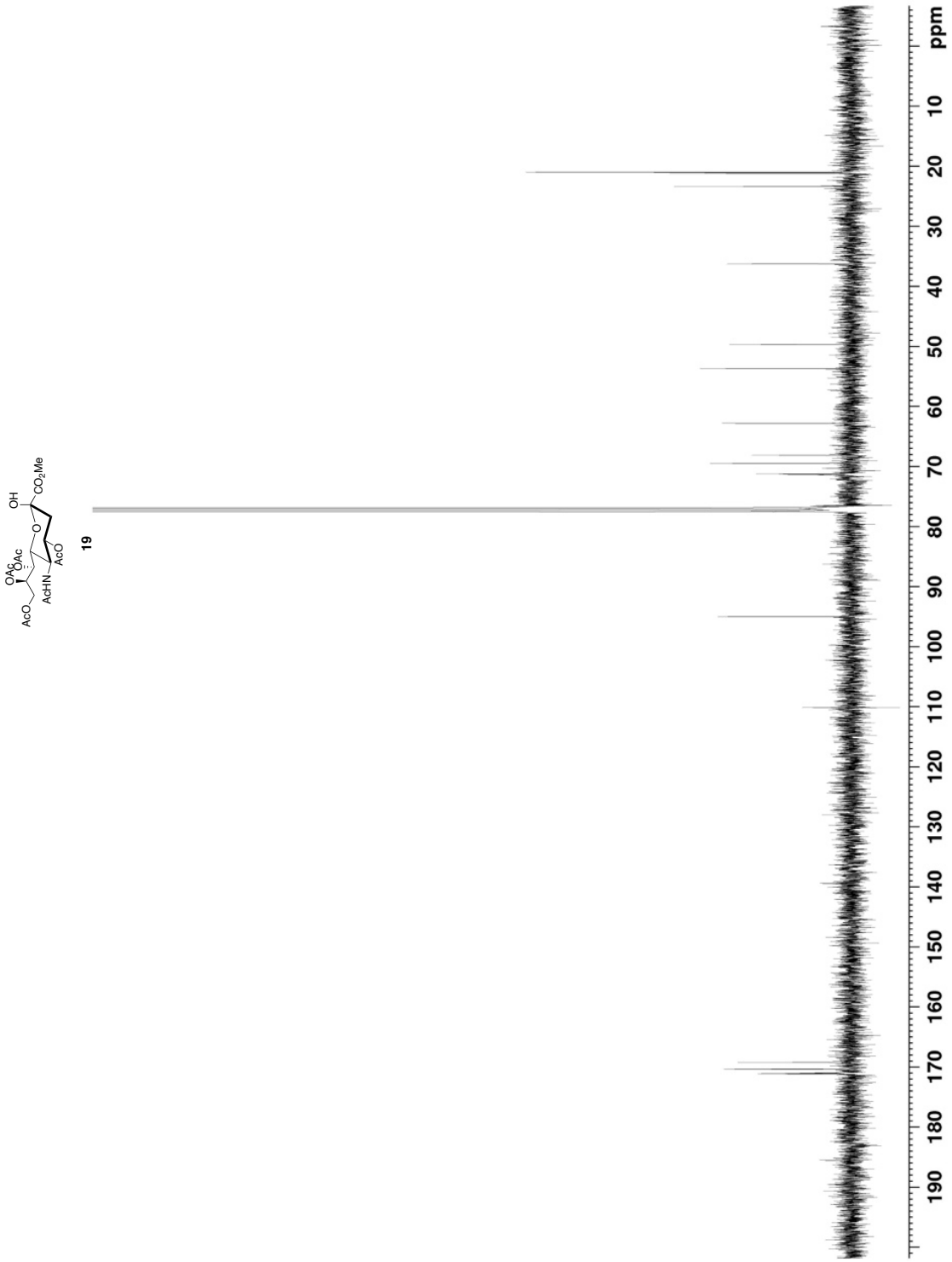


Figure A3. 20 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 19

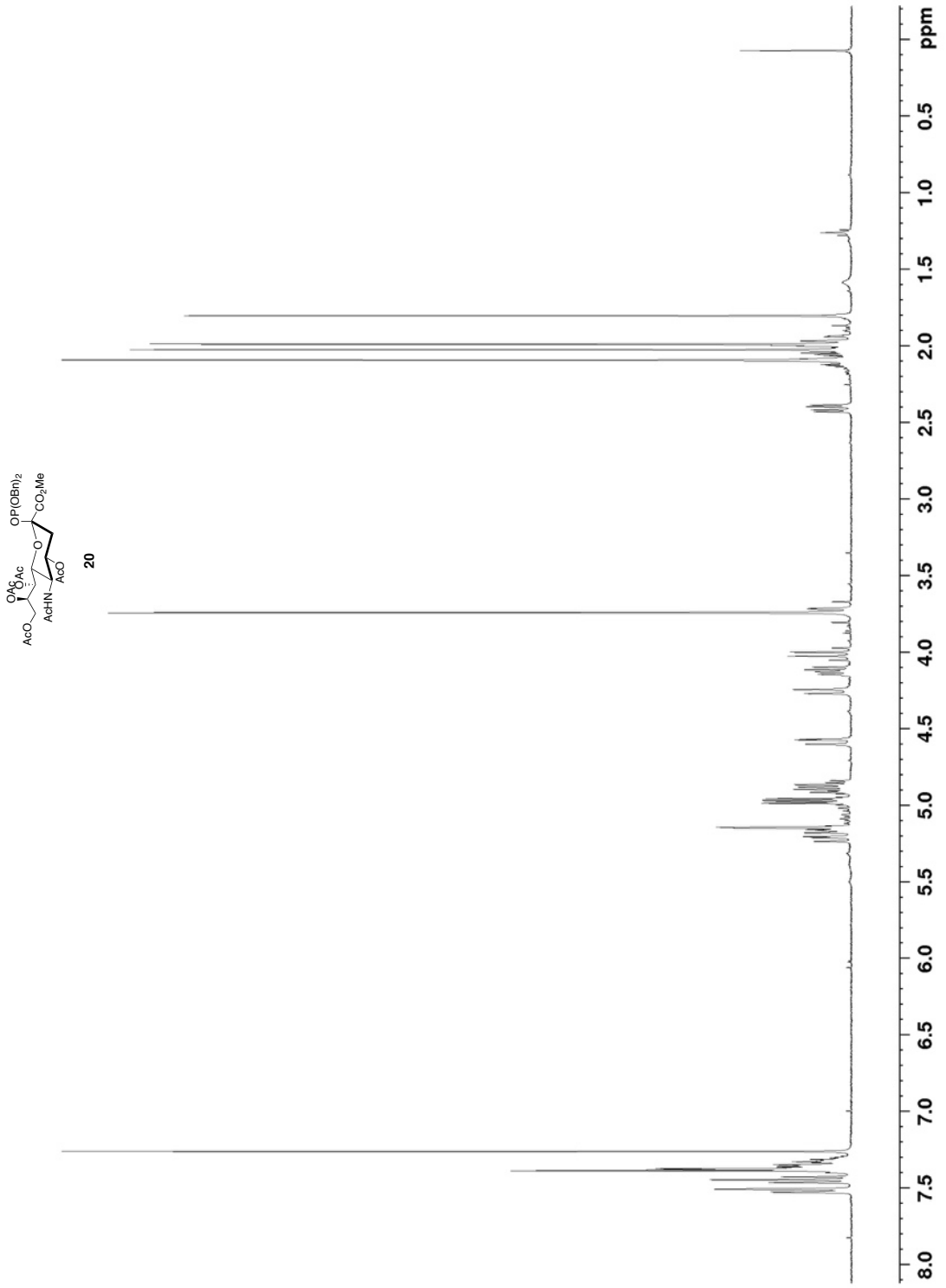


Figure A3. 21 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 20

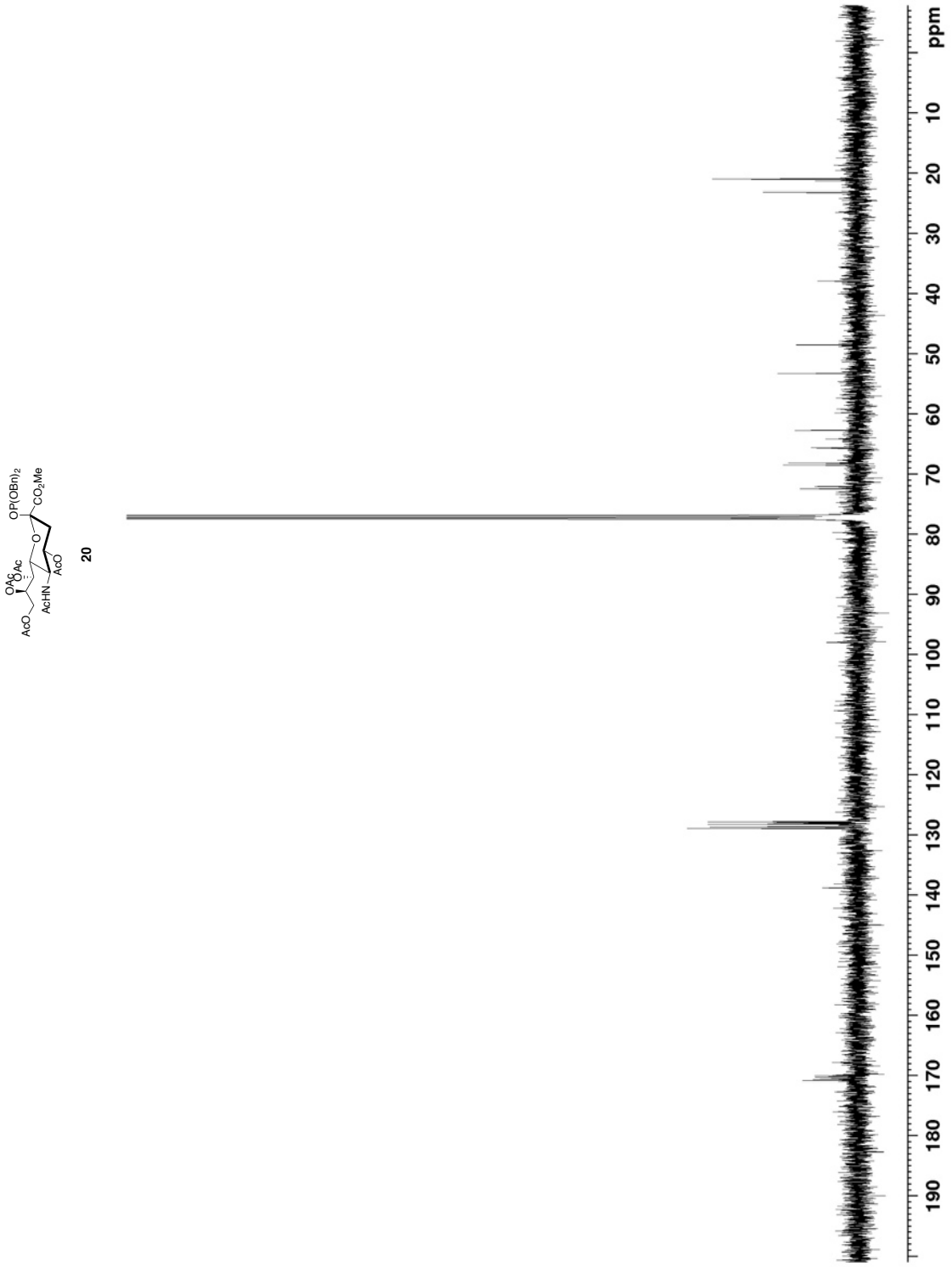


Figure A3. 22 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 20

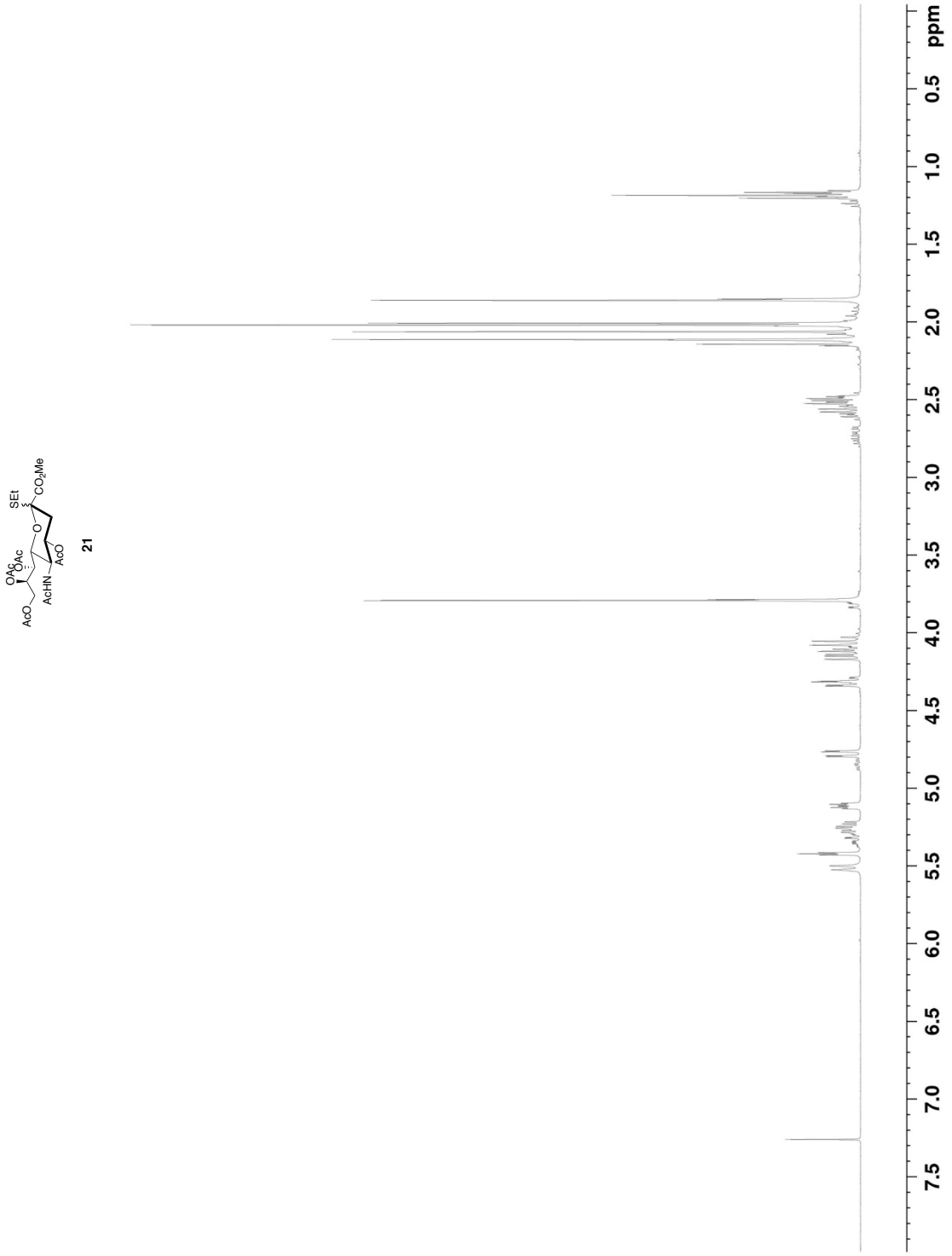


Figure A3. 23 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 21

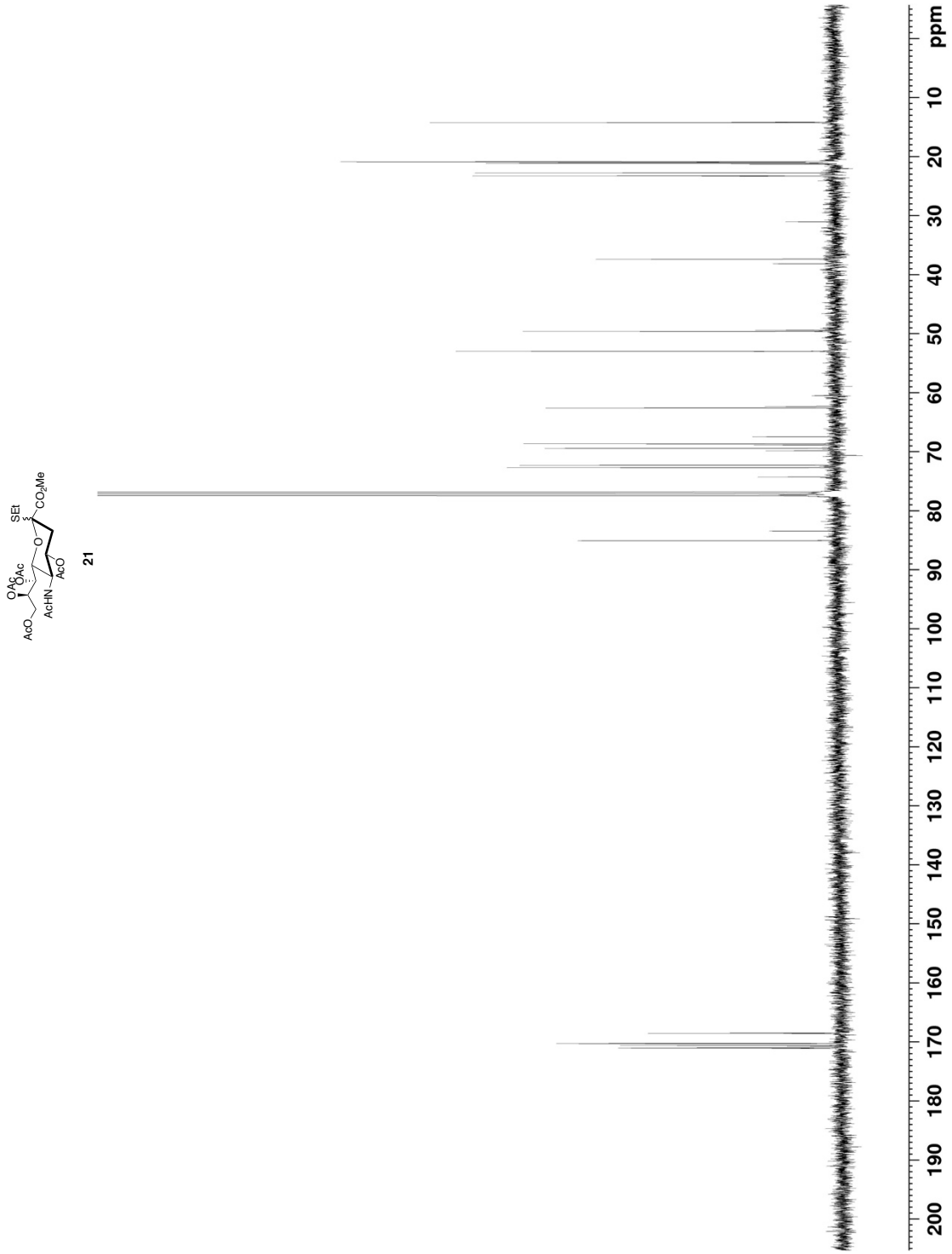


Figure A3. 24 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 21

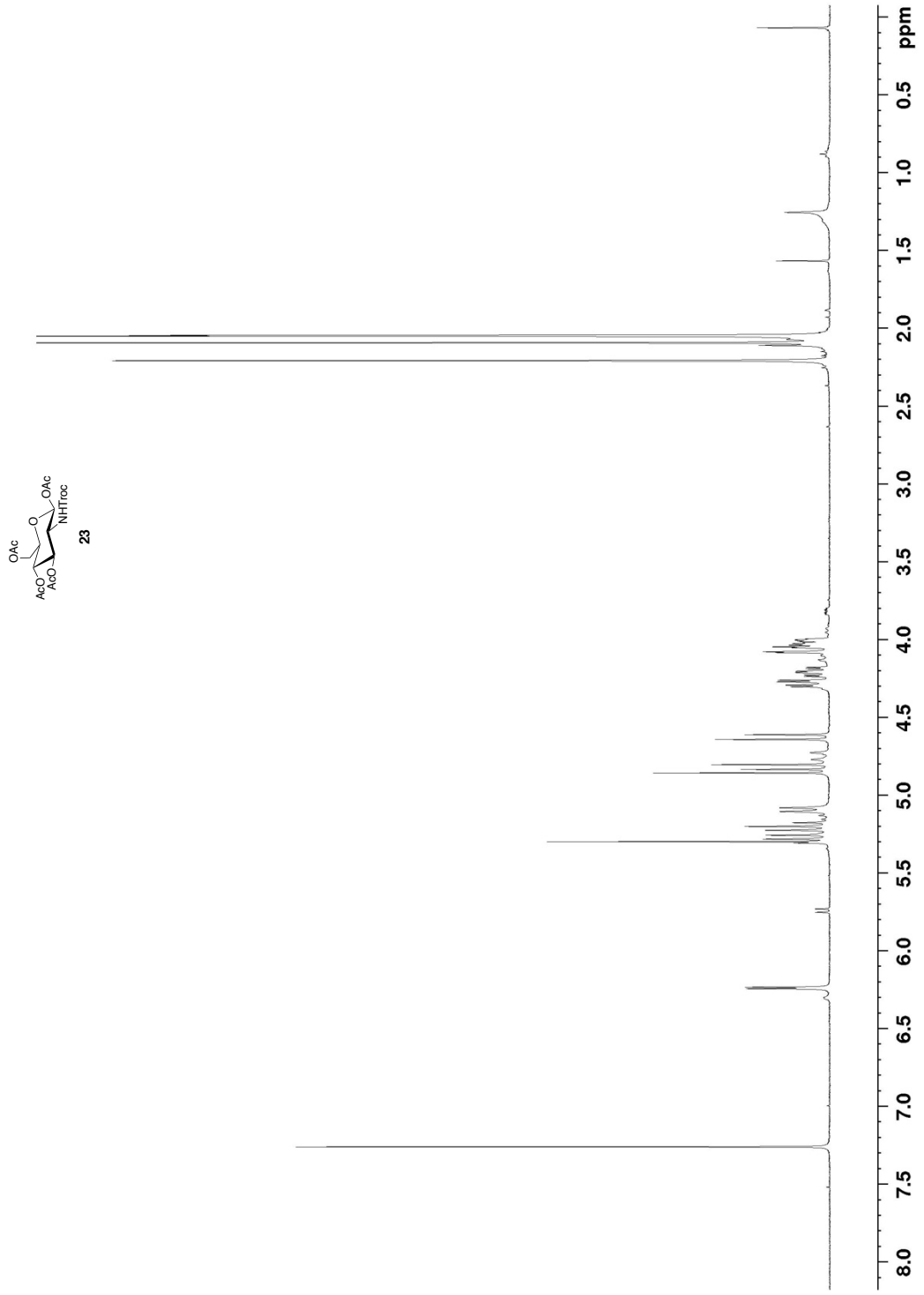


Figure A3. 25 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 23

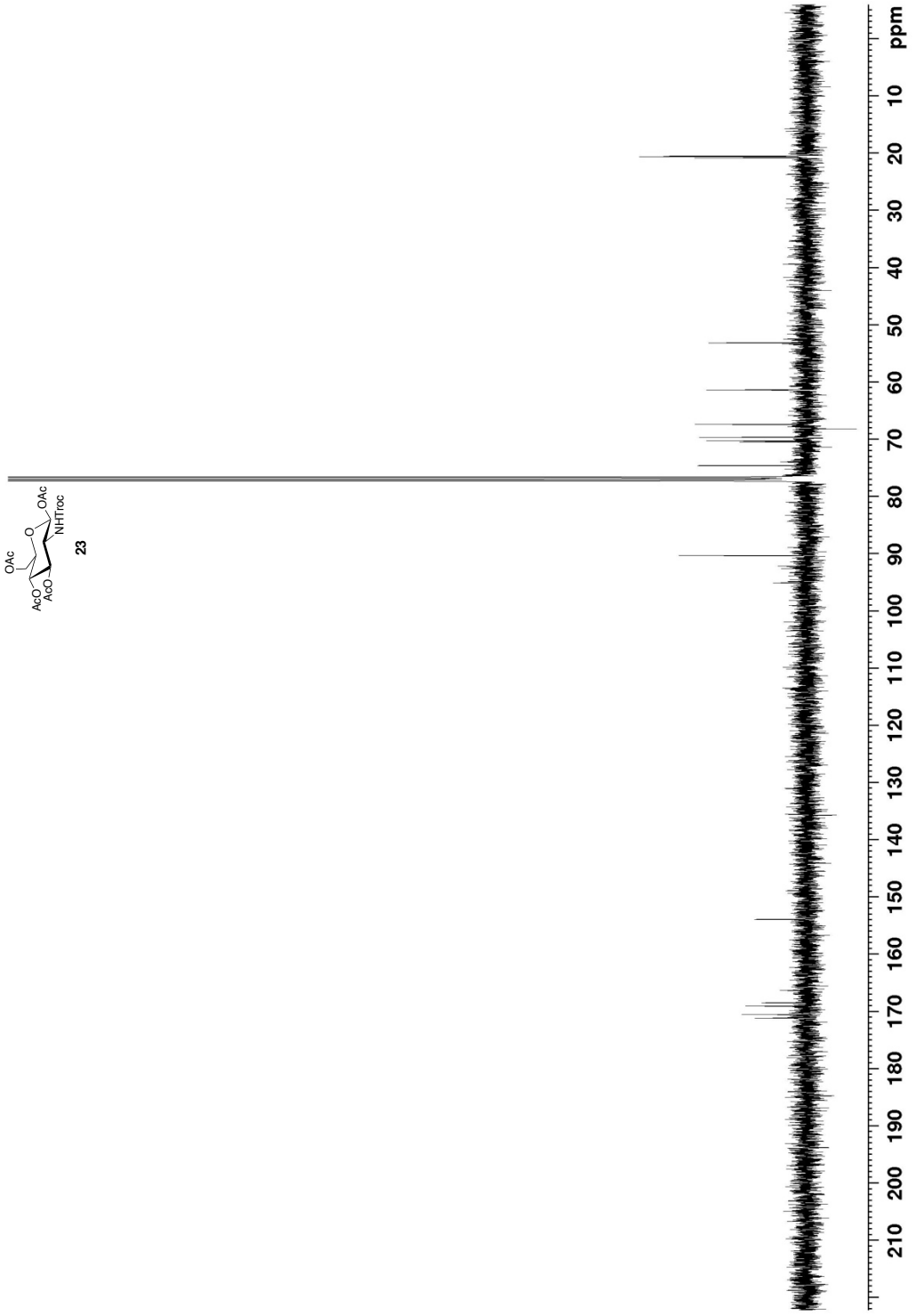


Figure A3. 26 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 23

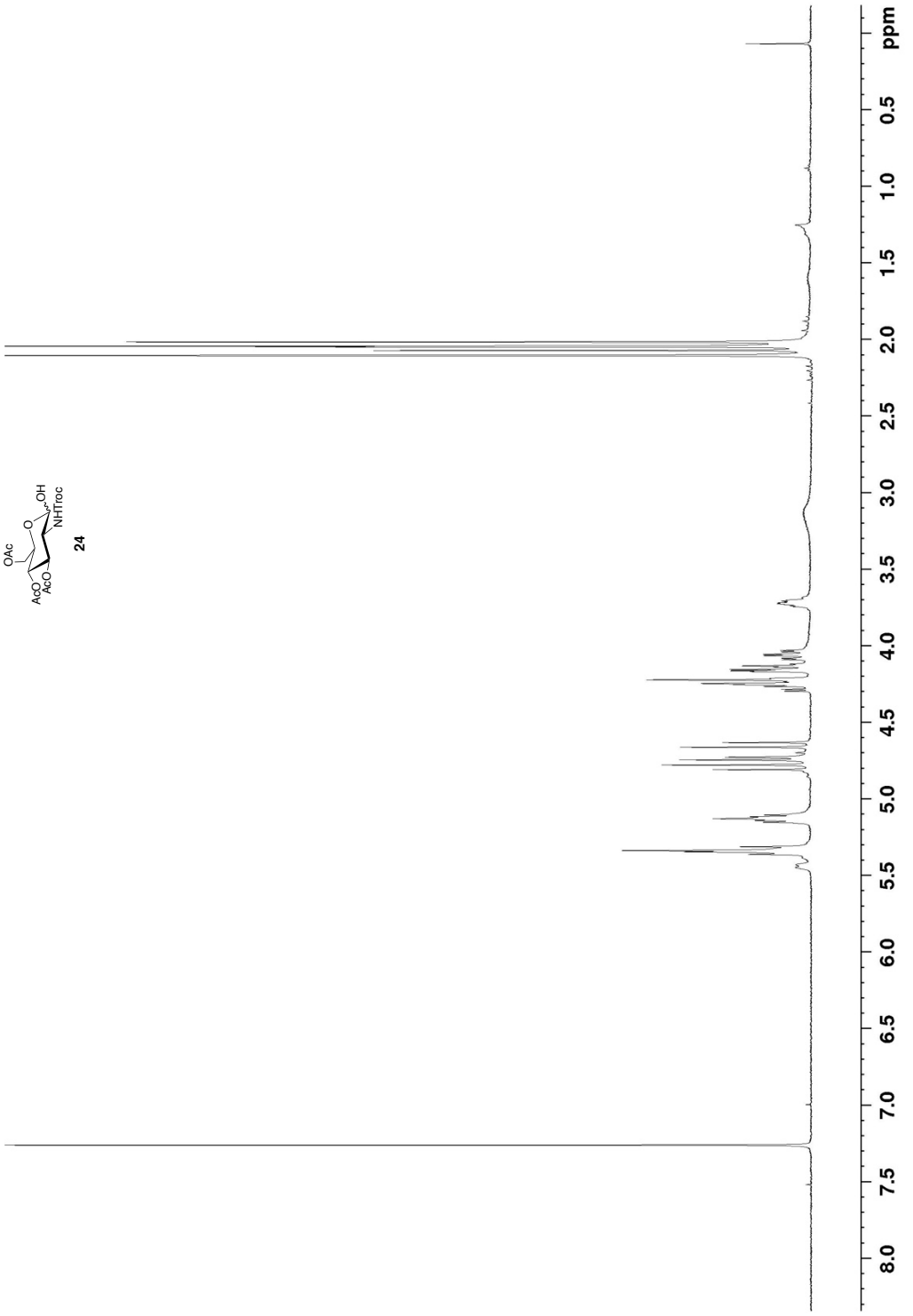


Figure A3. 27 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 24

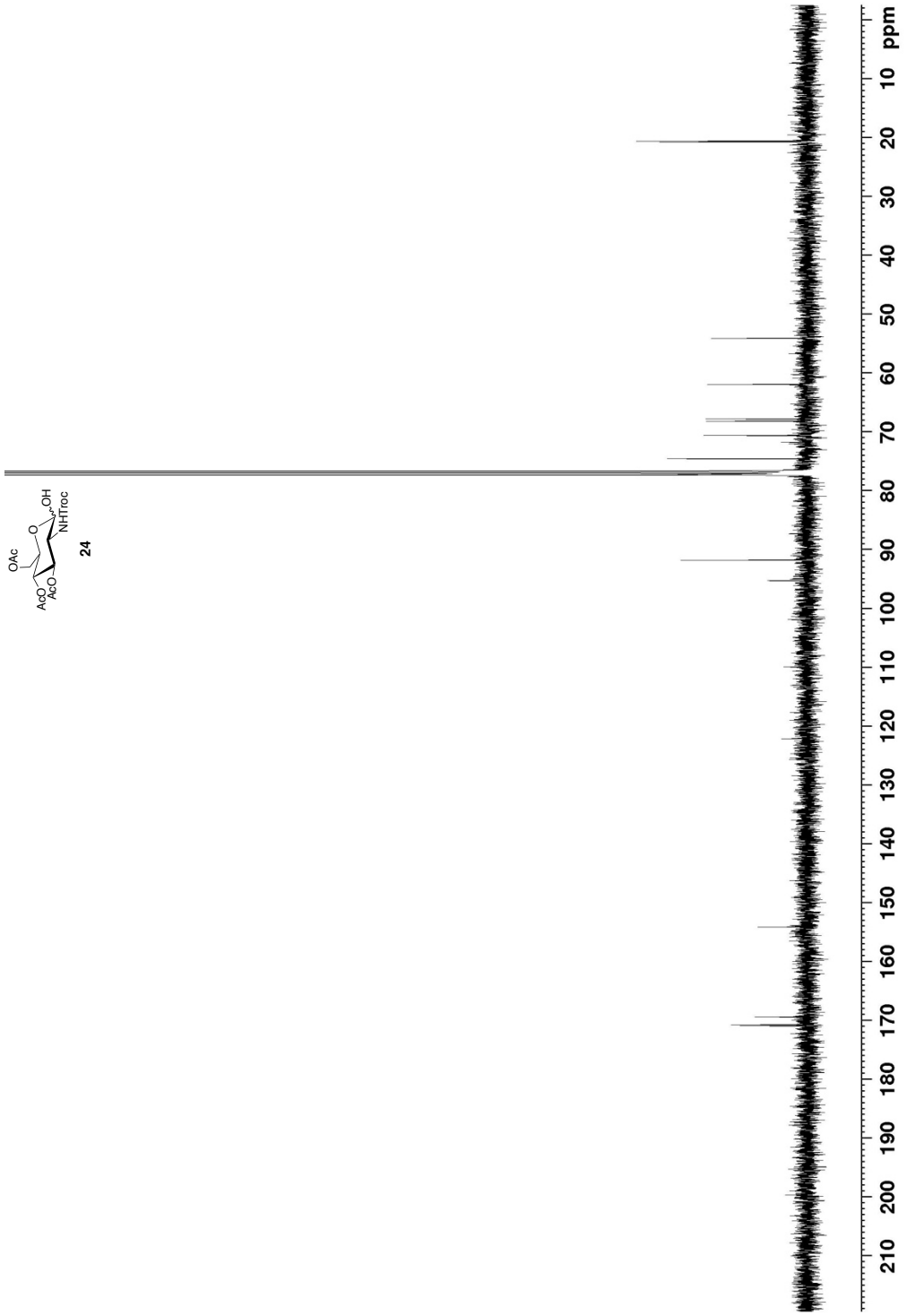


Figure A3. 28 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 24

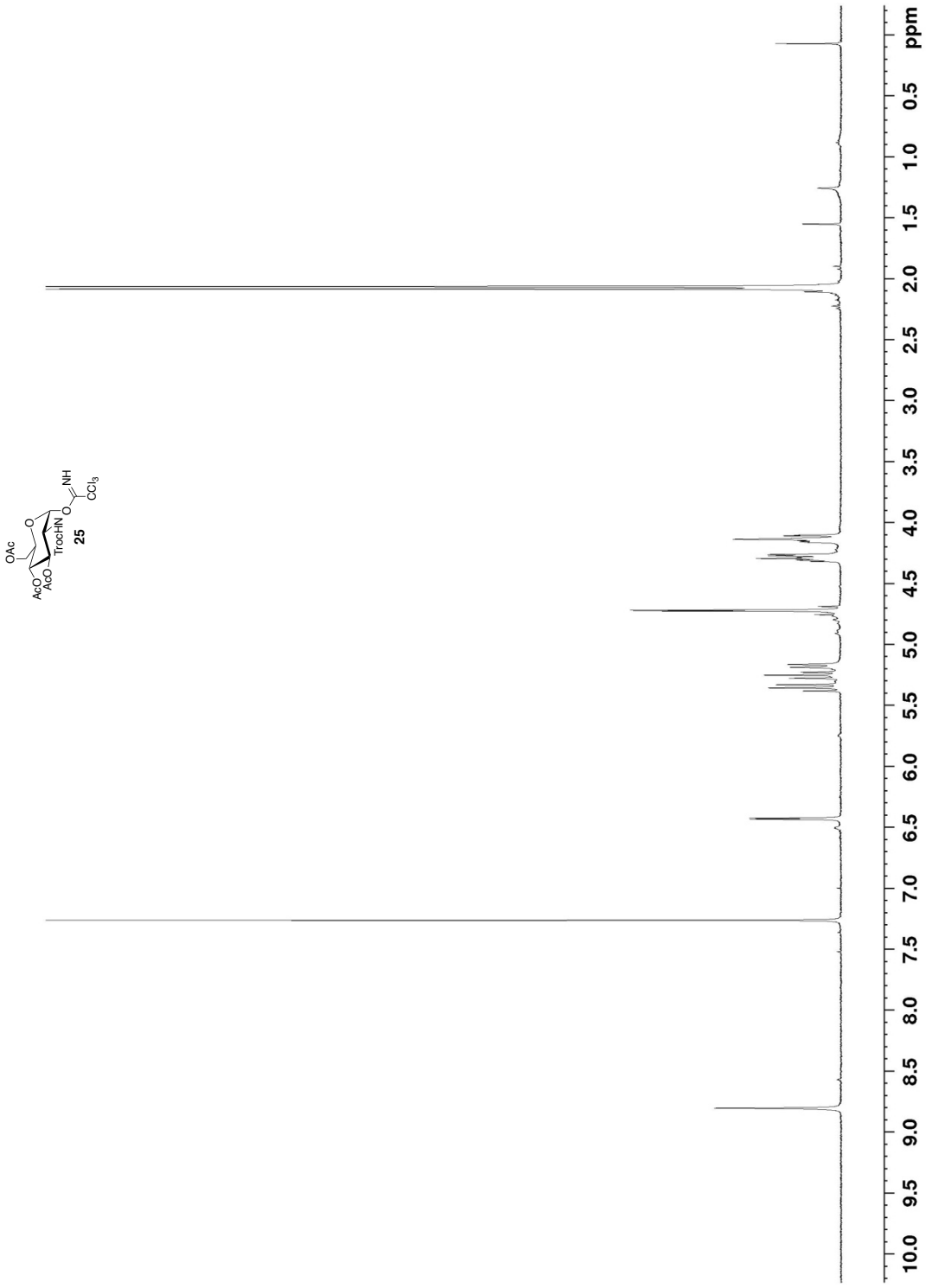


Figure A3. 29 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 25

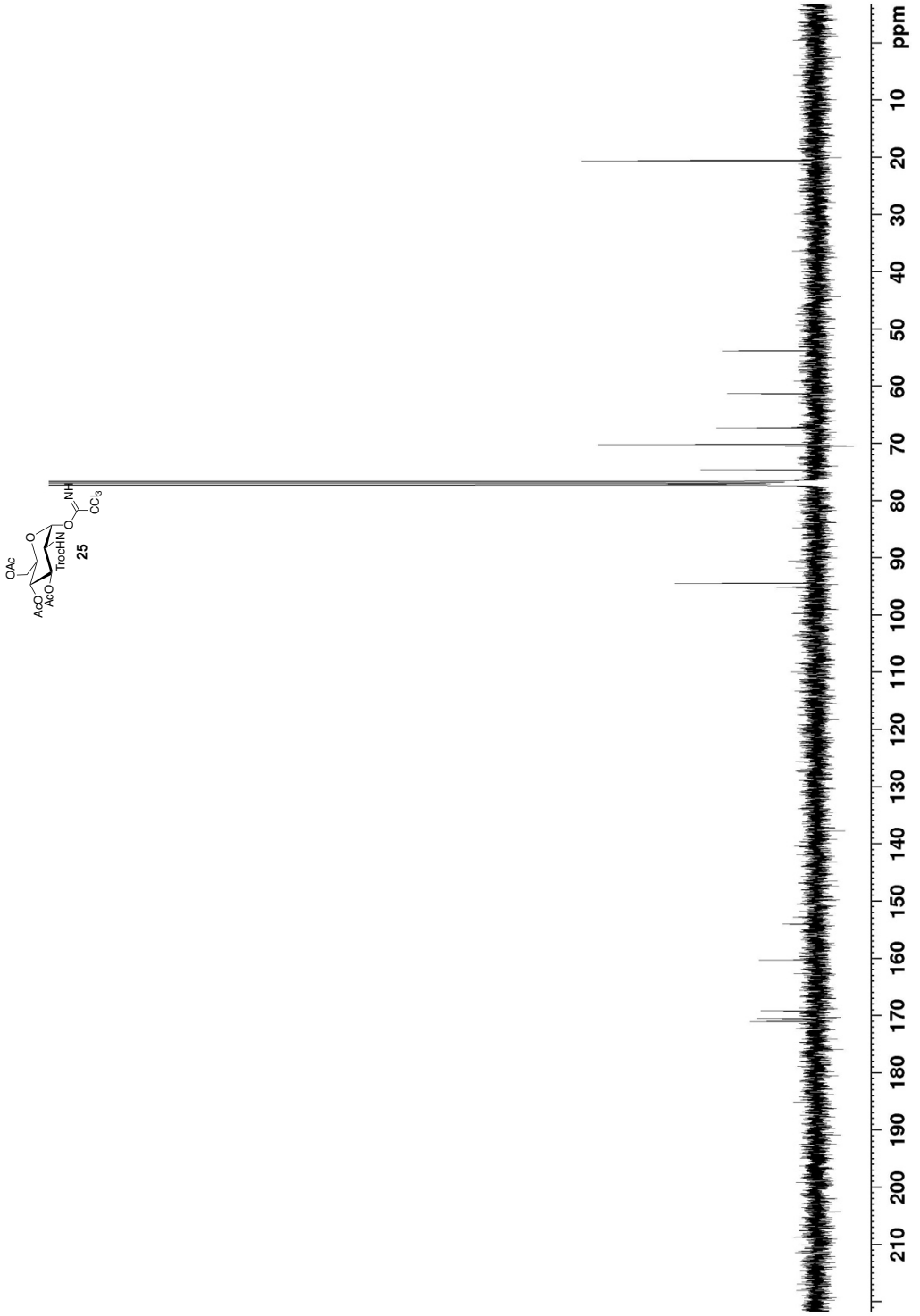


Figure A3. 30 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 25

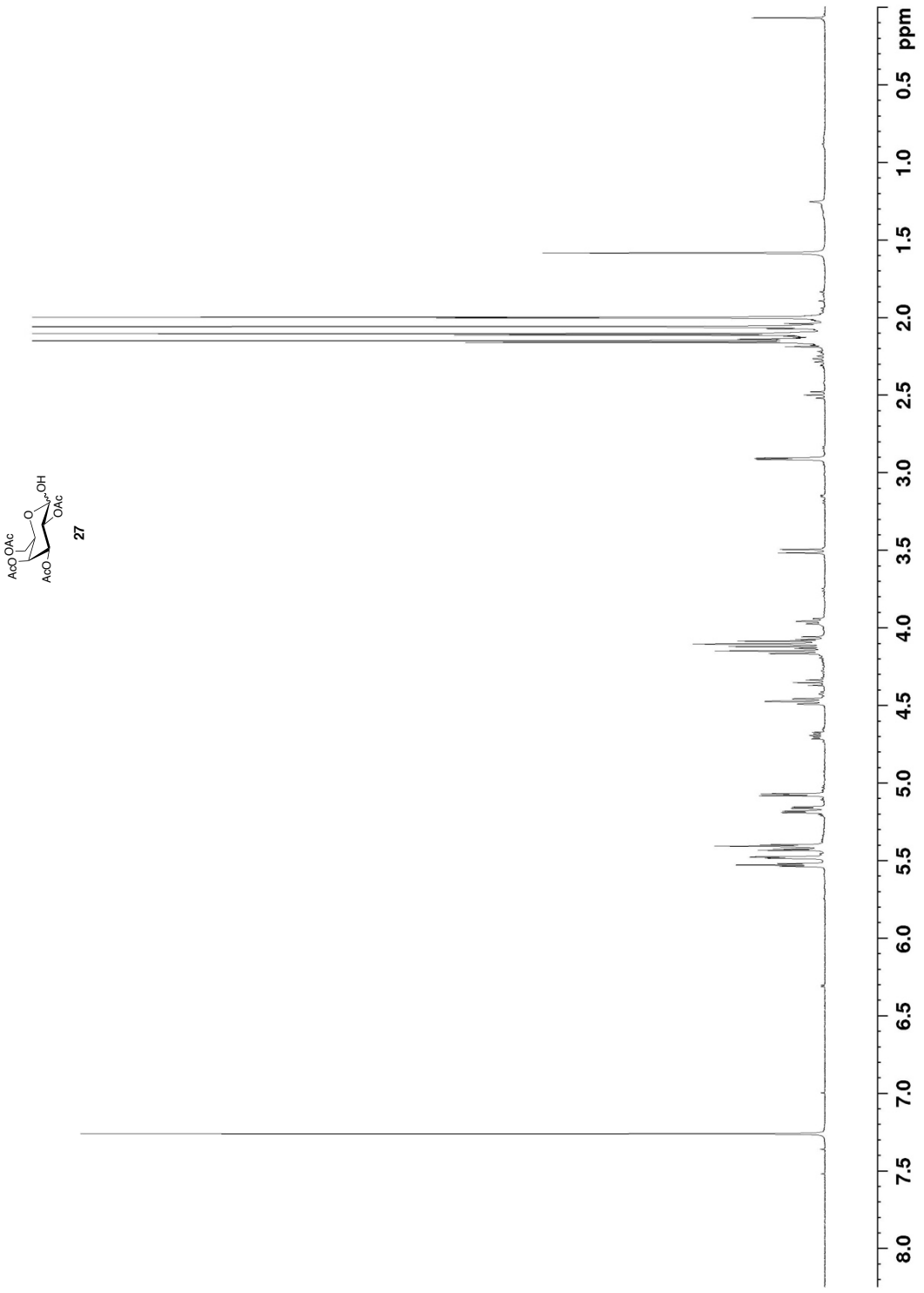


Figure A3. 31 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 27

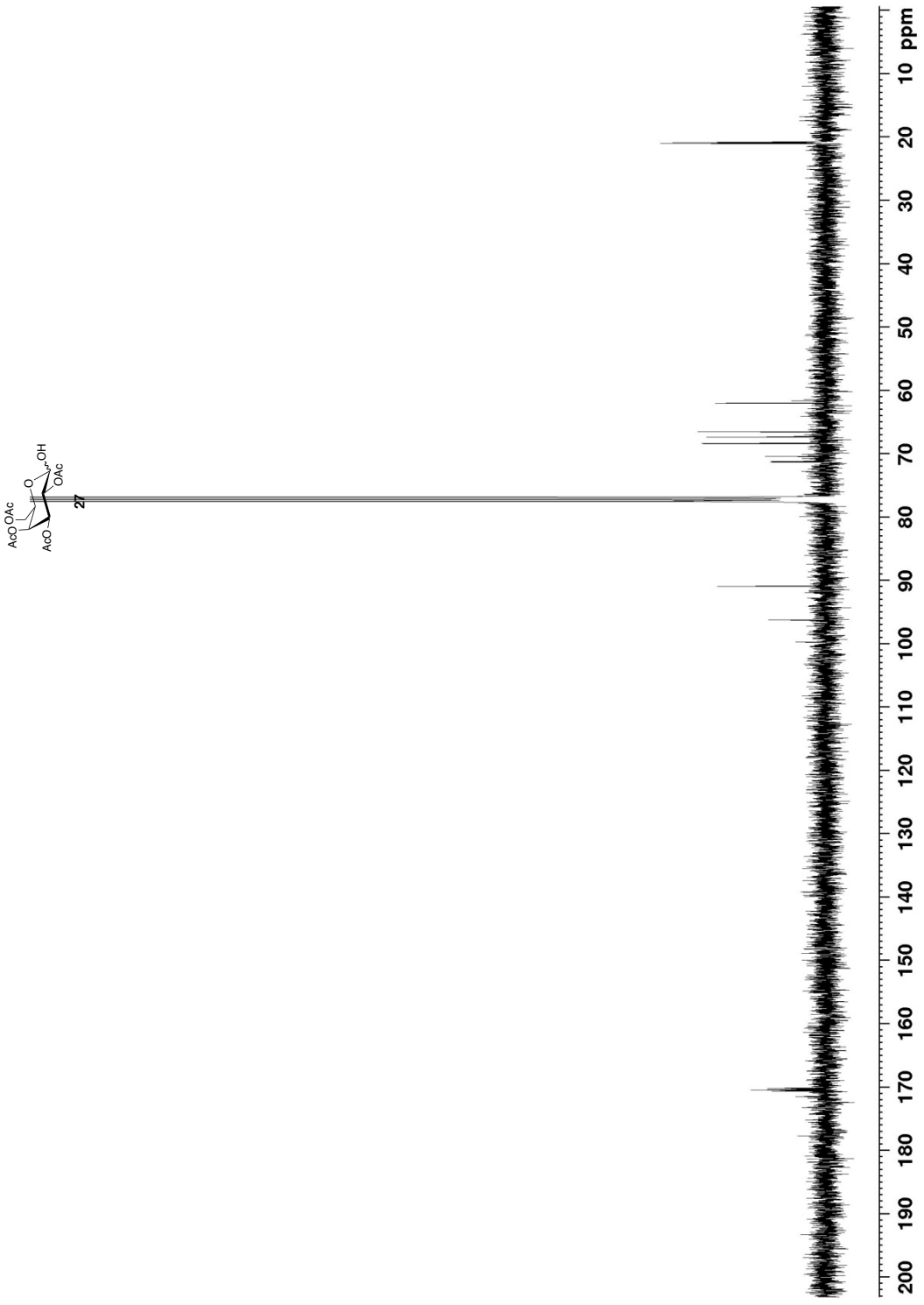


Figure A3. 32 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 27

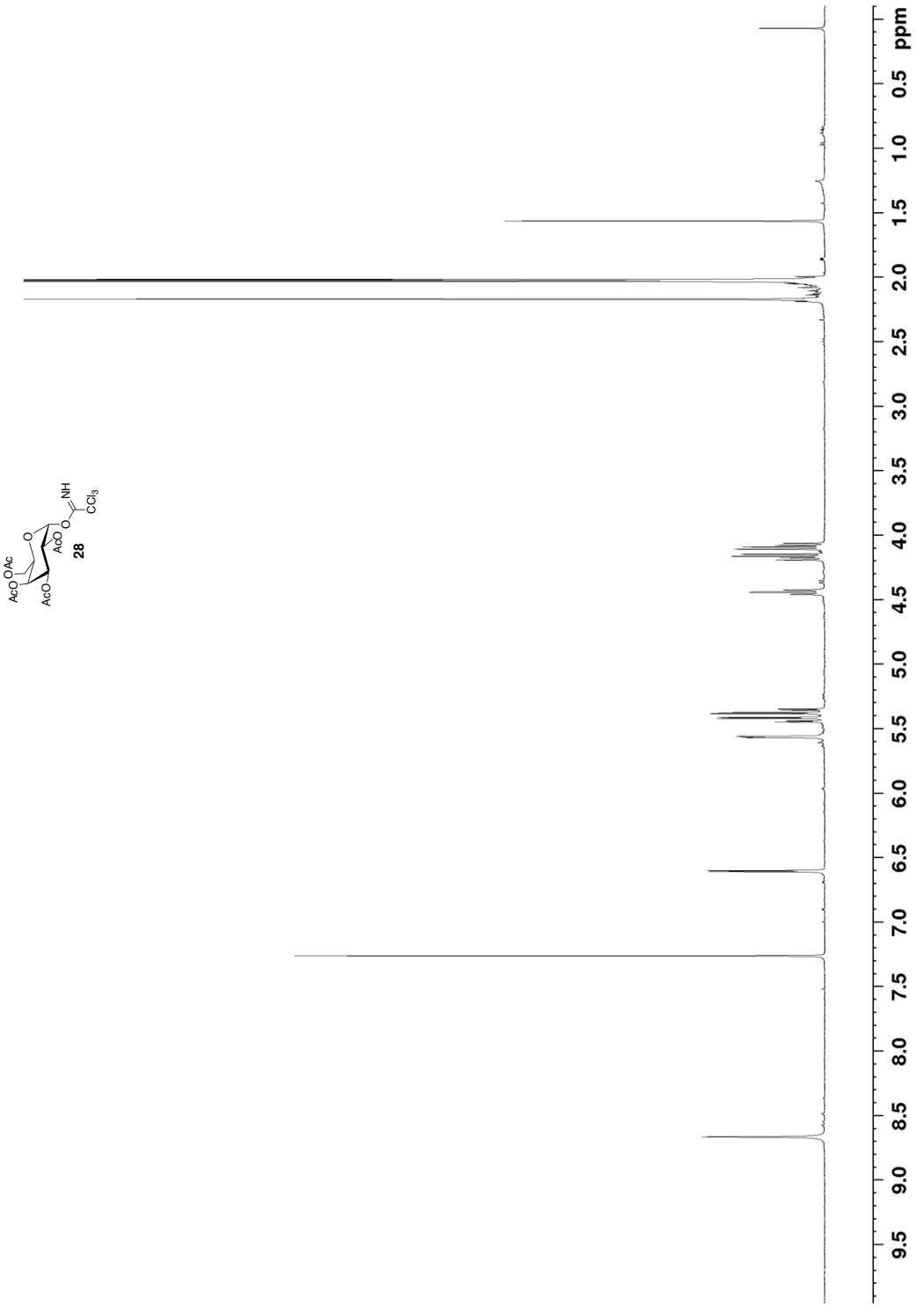


Figure A3. 33 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 28

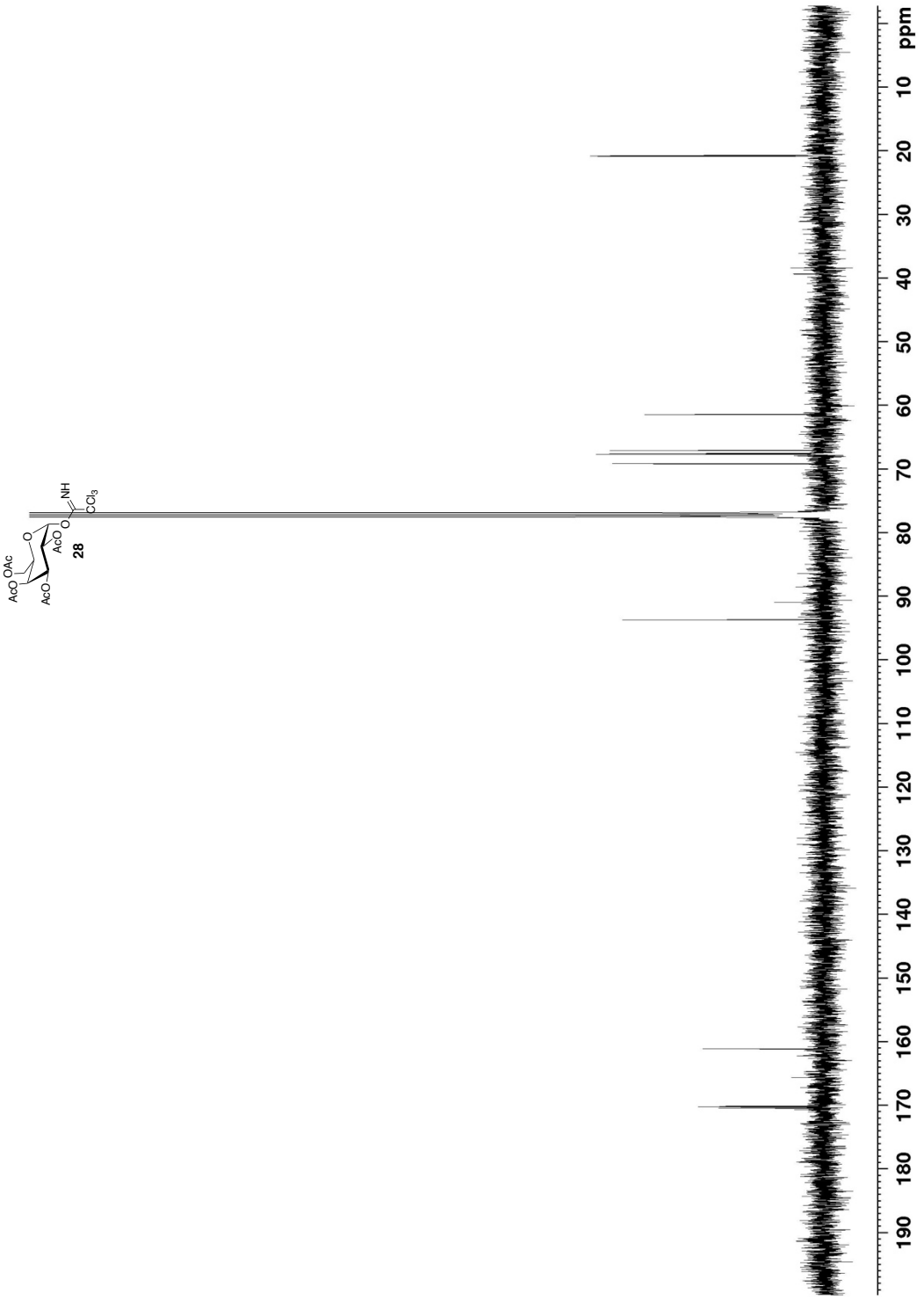


Figure A3. 34 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 28

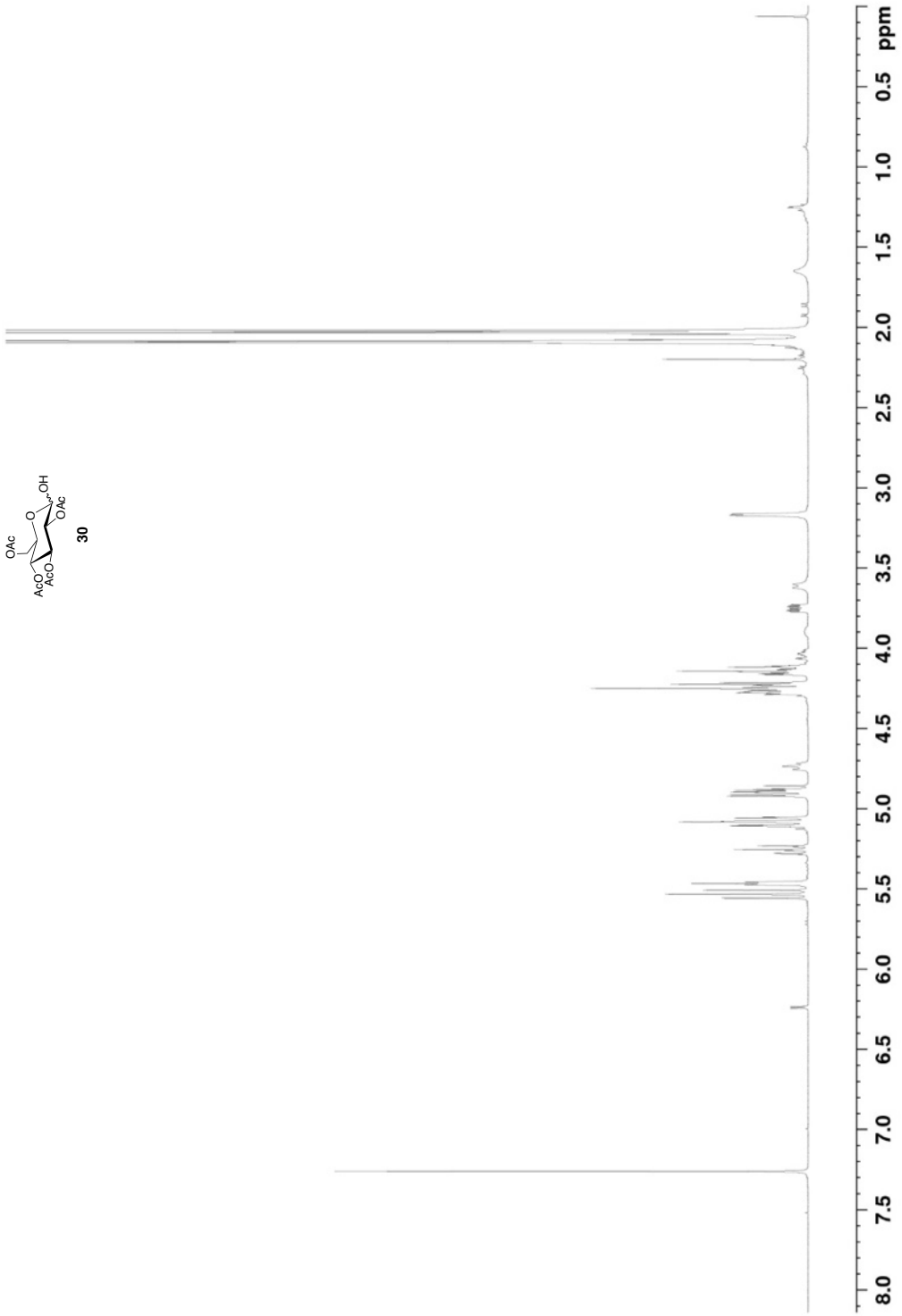


Figure A3. 35 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 30

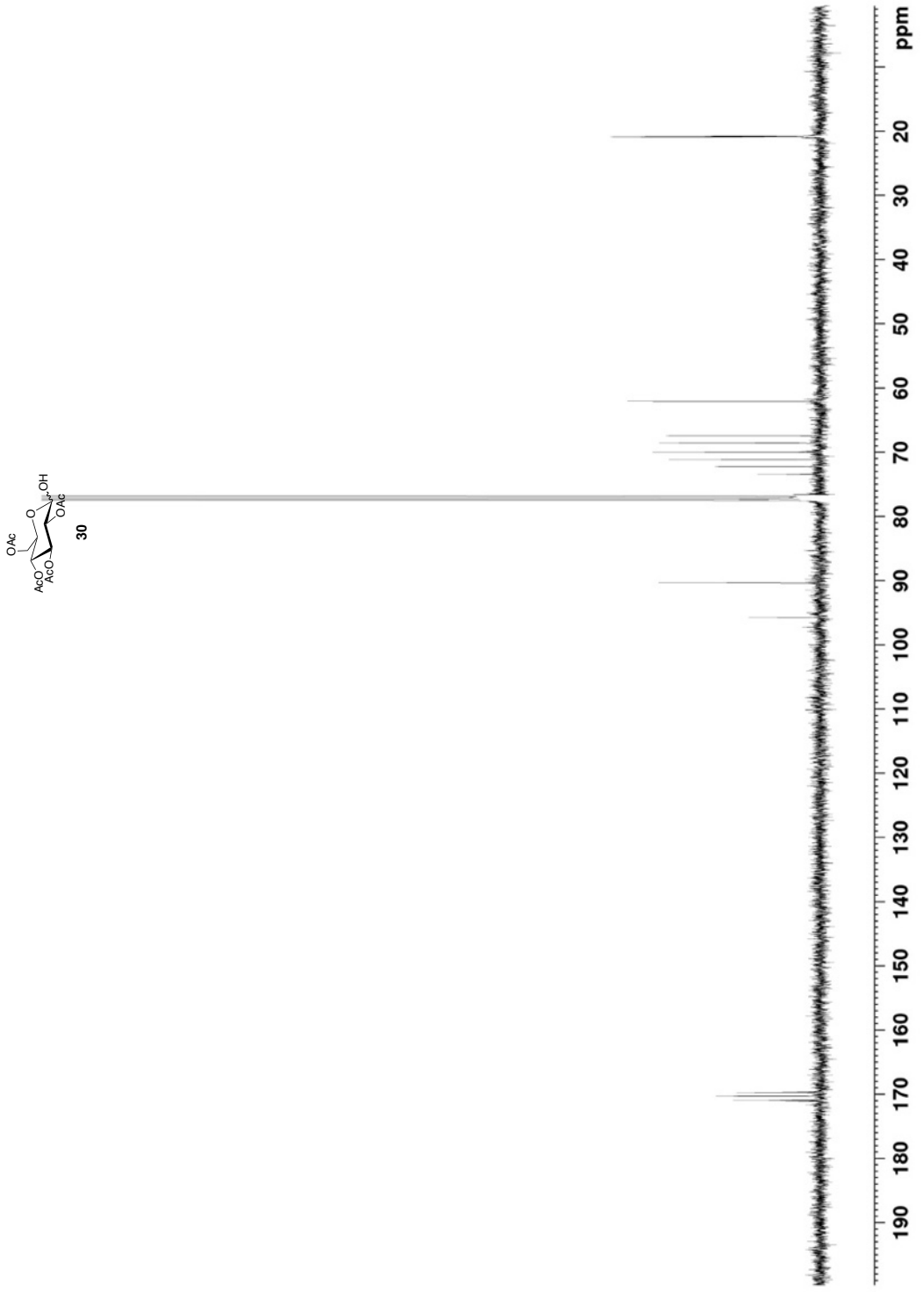


Figure A3. 36 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 30

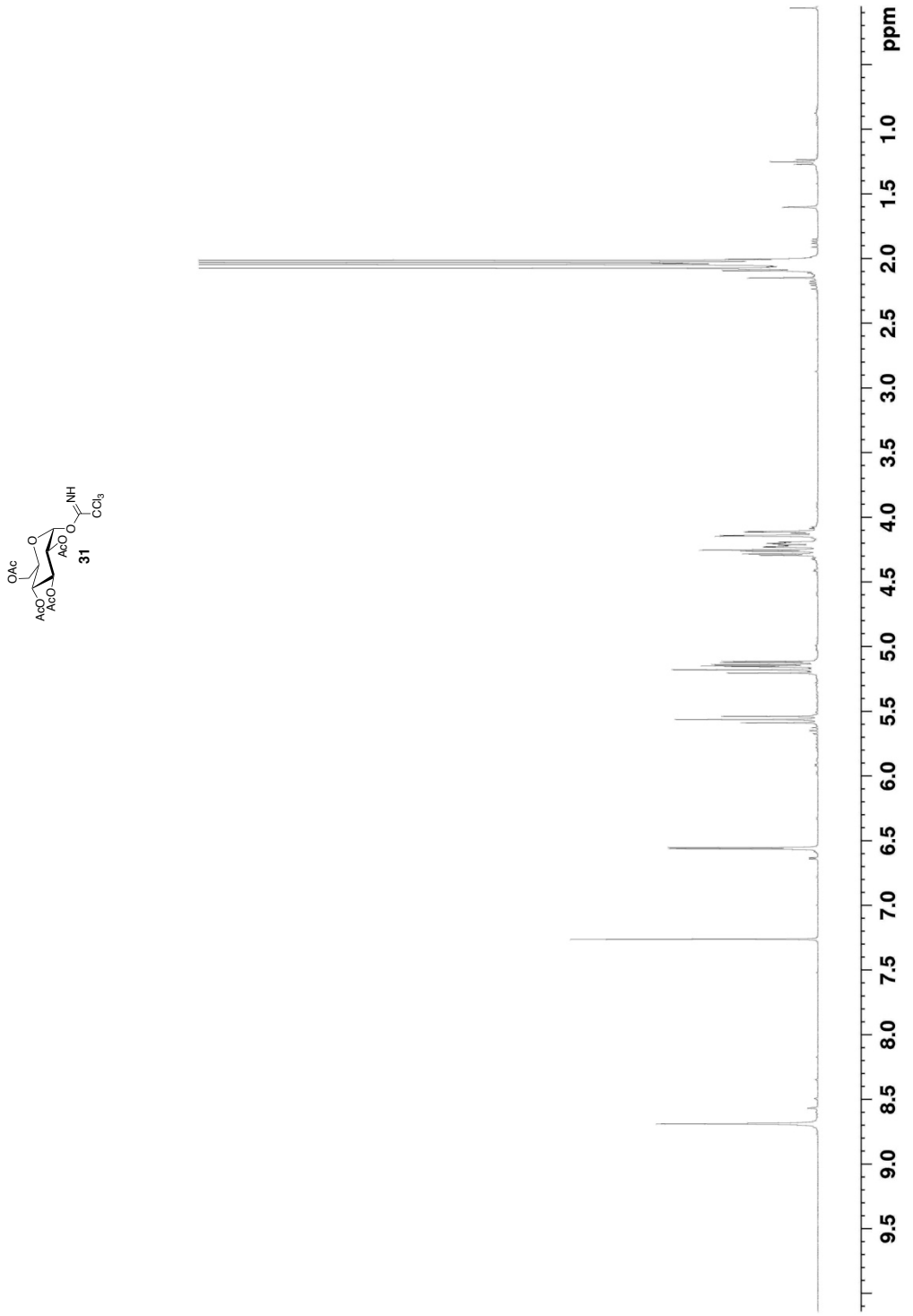


Figure A3. 37 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 31

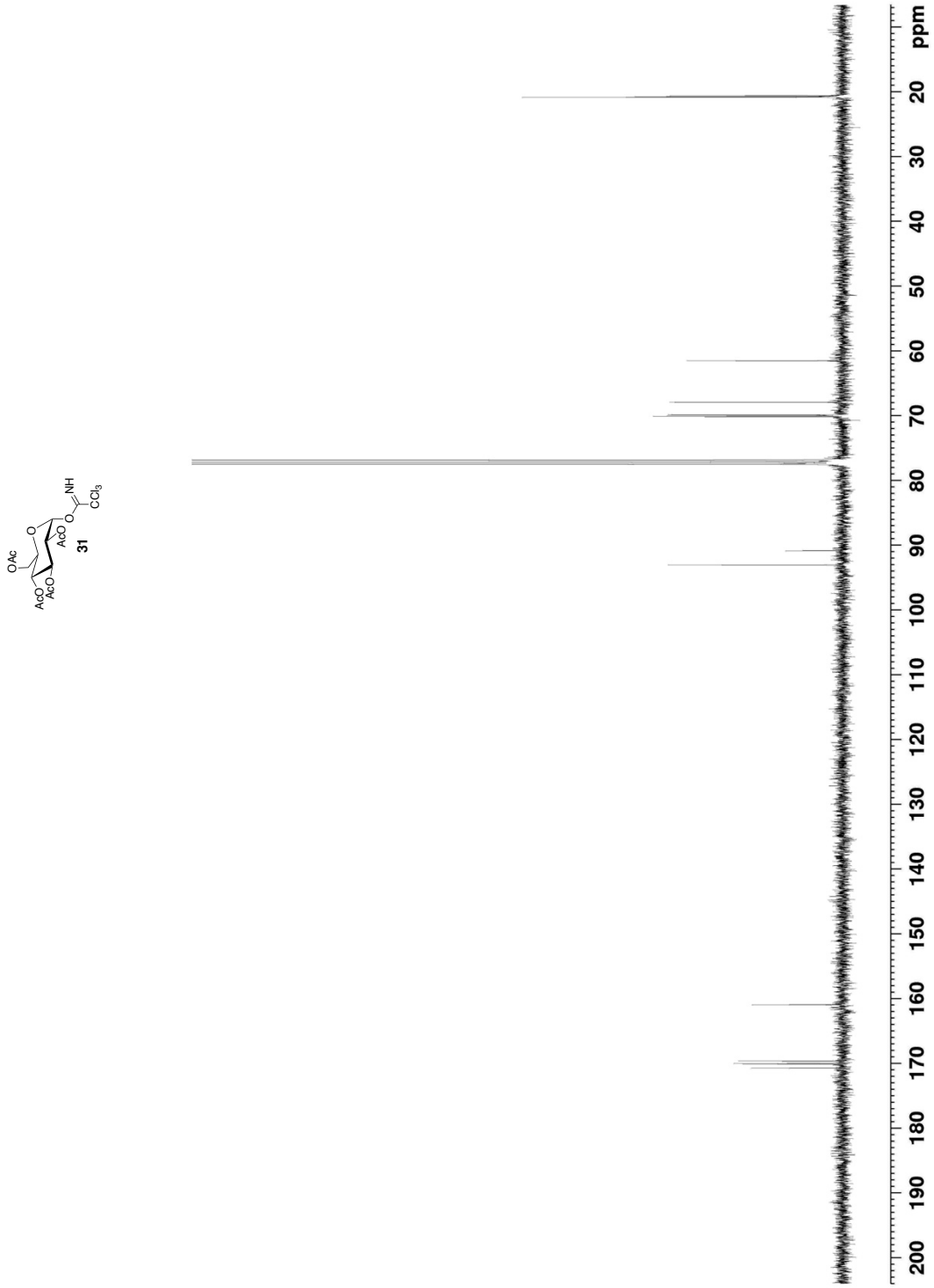


Figure A3. 38 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 31

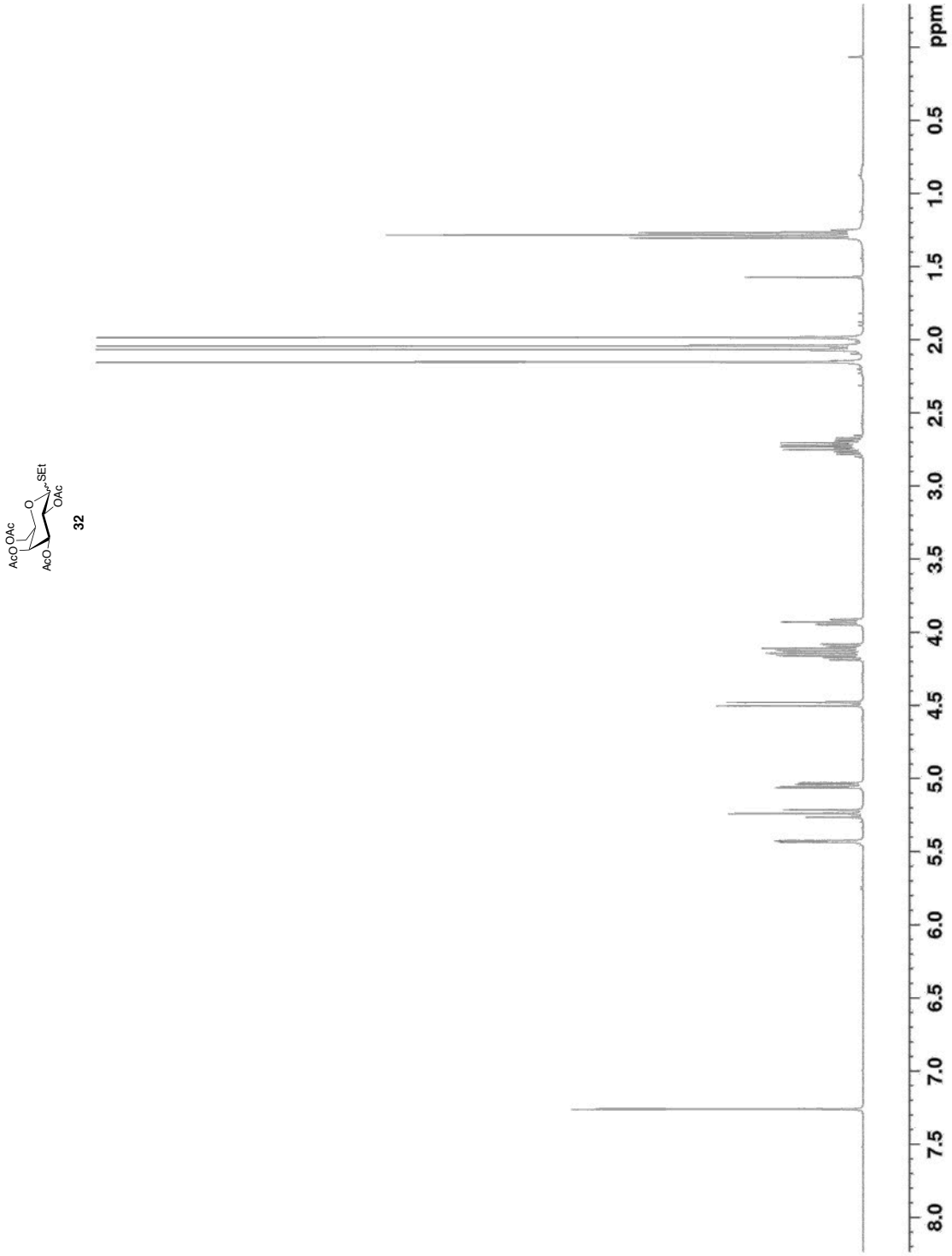


Figure A3. 39 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 32

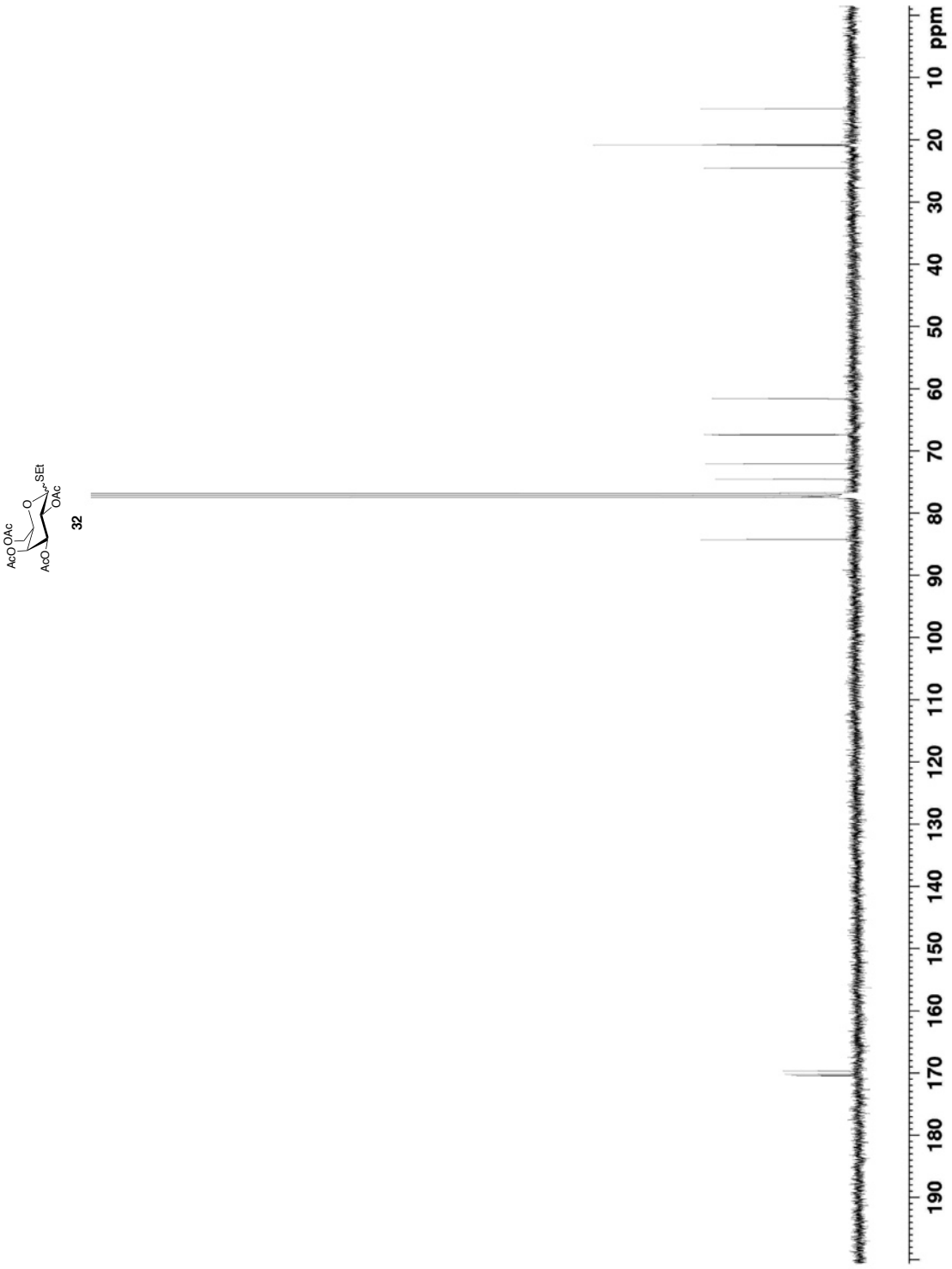


Figure A3. 40 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 32

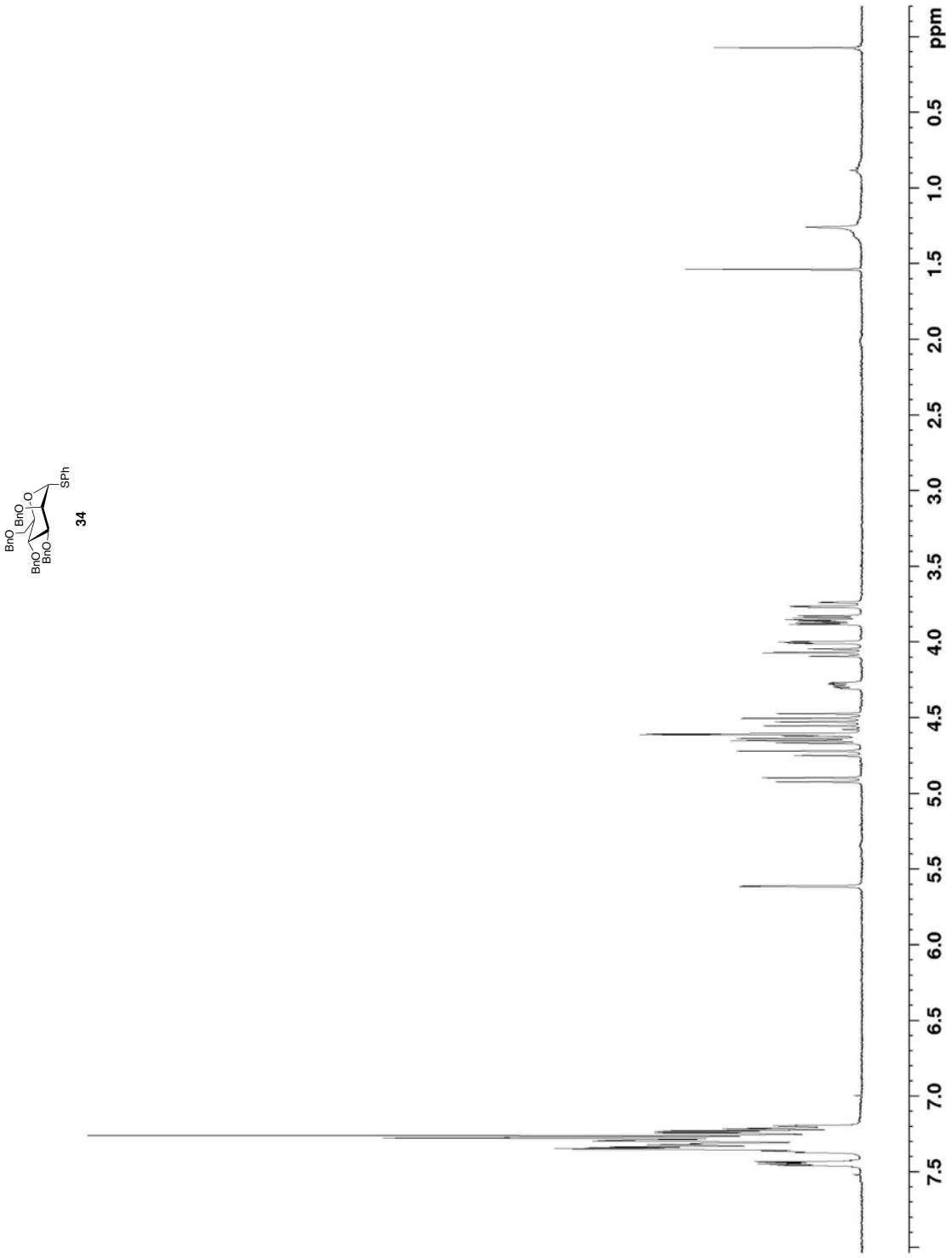


Figure A3. 41 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 34

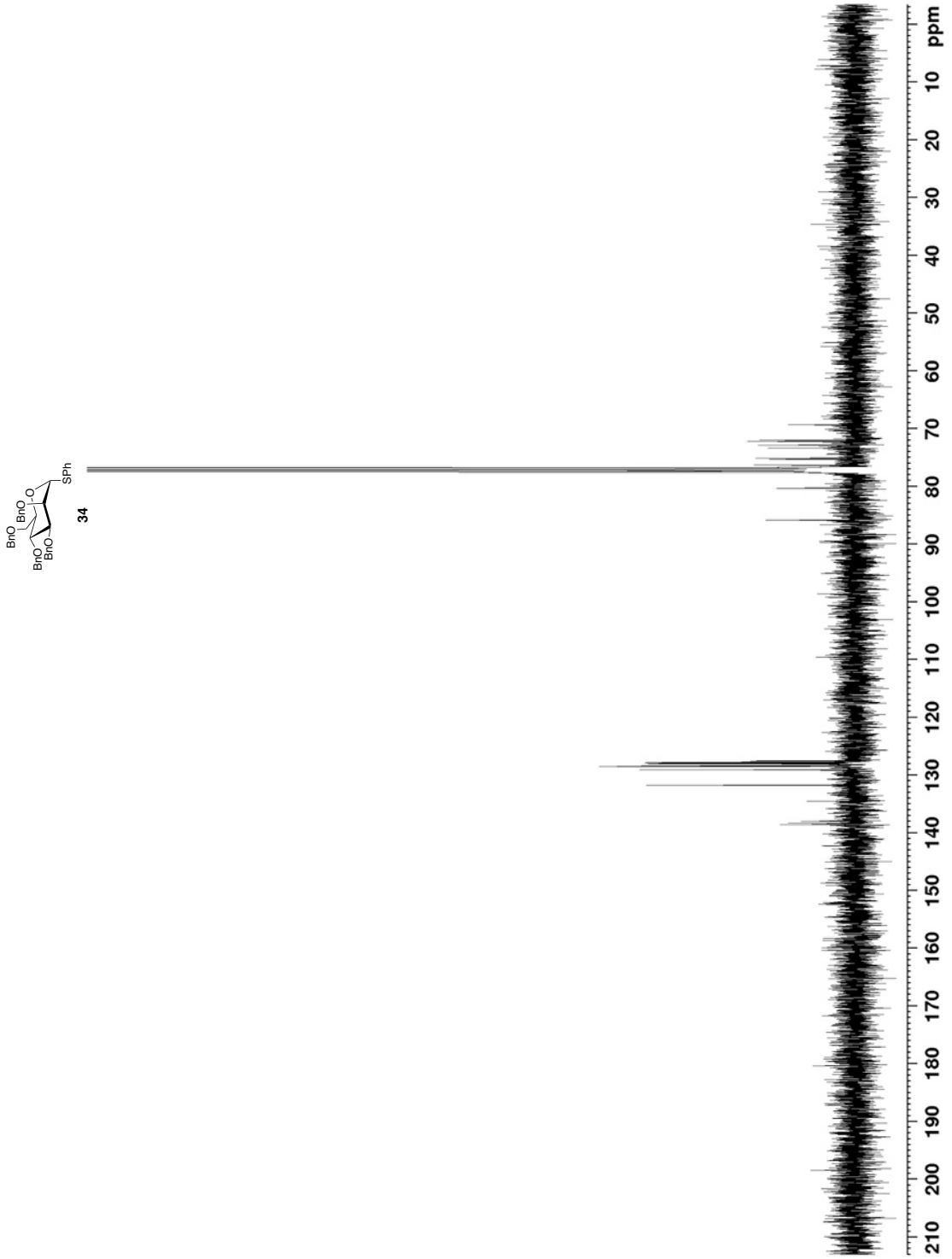


Figure A3. 42 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 34

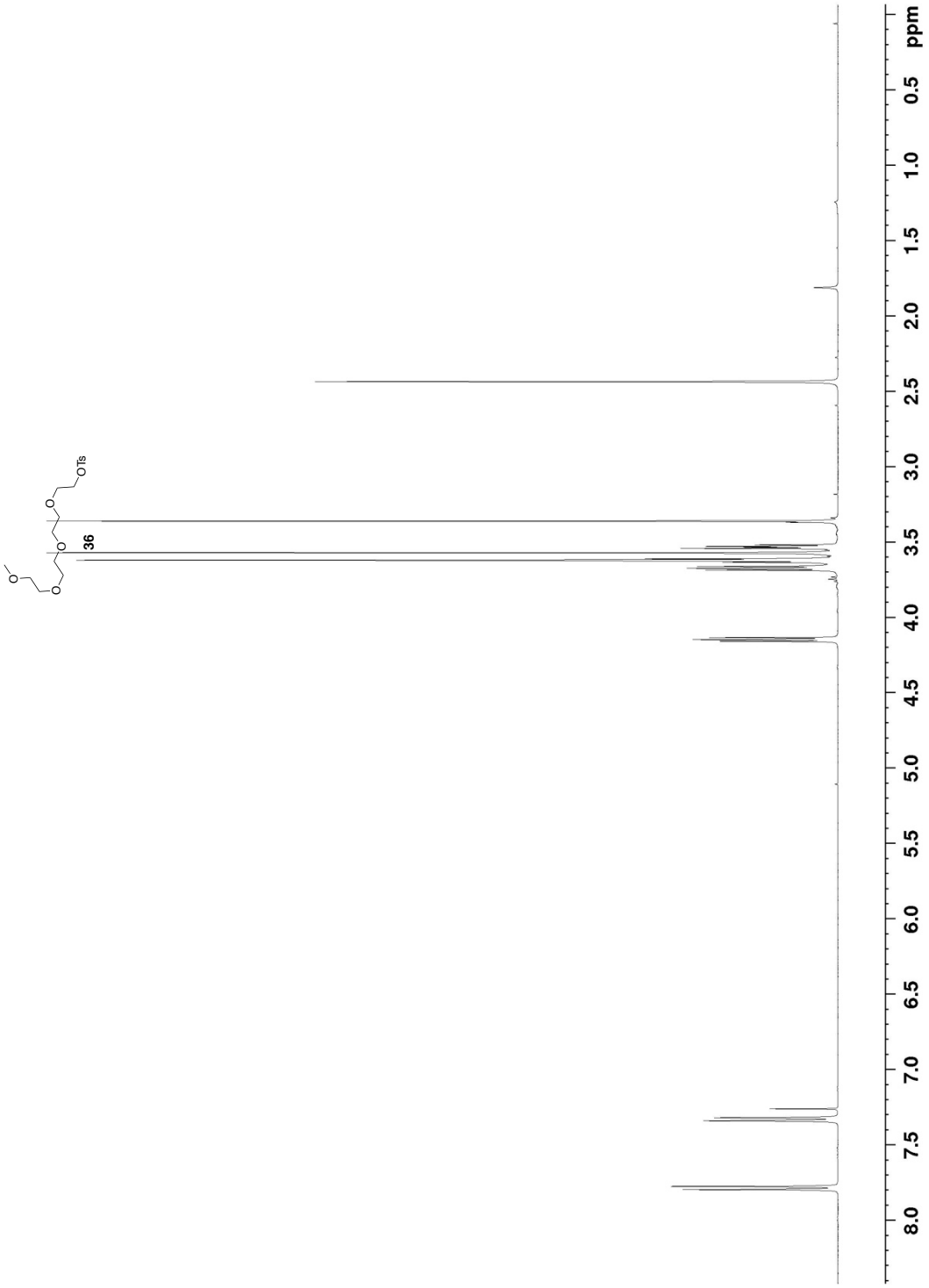


Figure A3. 43 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 36

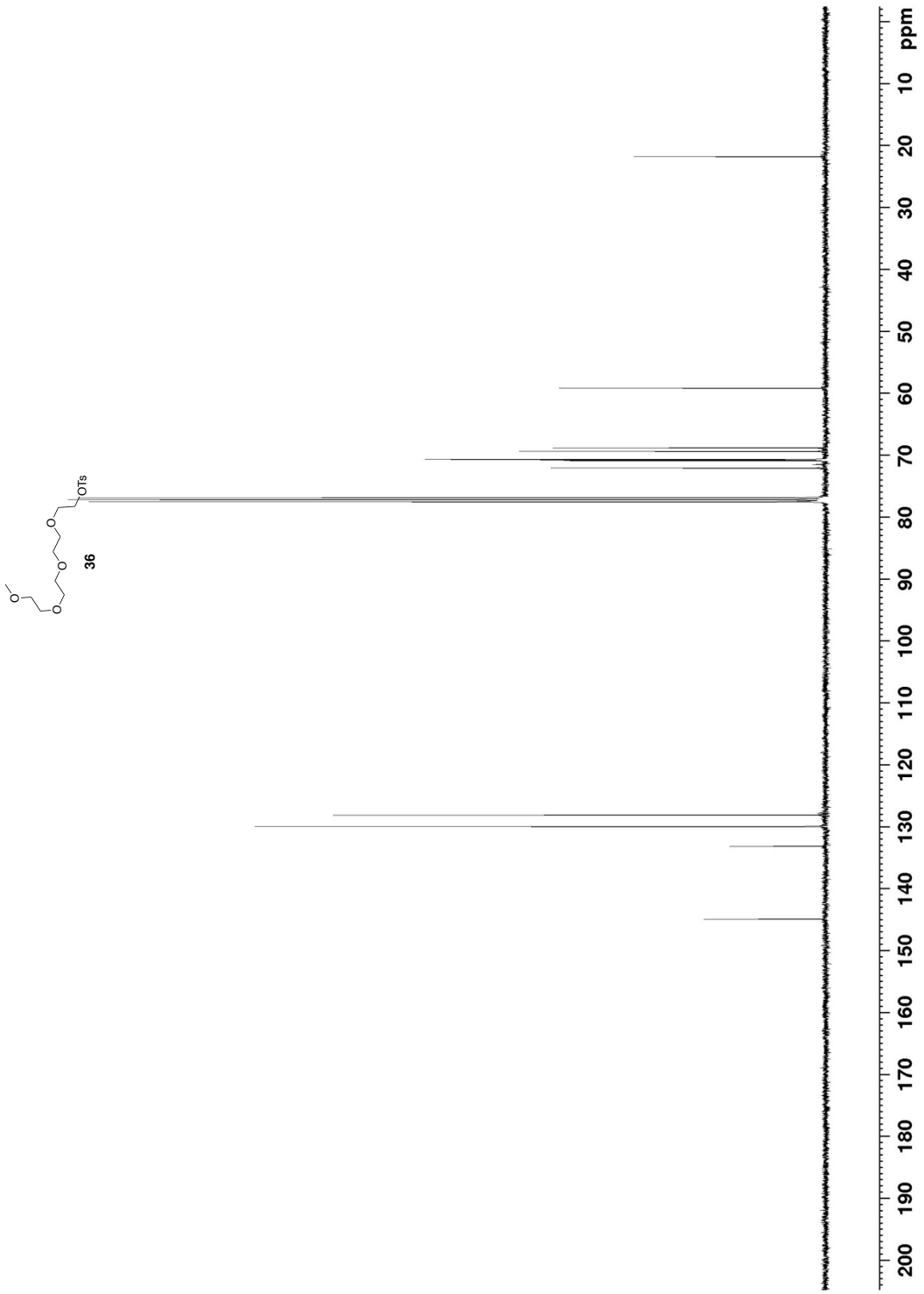


Figure A3. 44 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 36

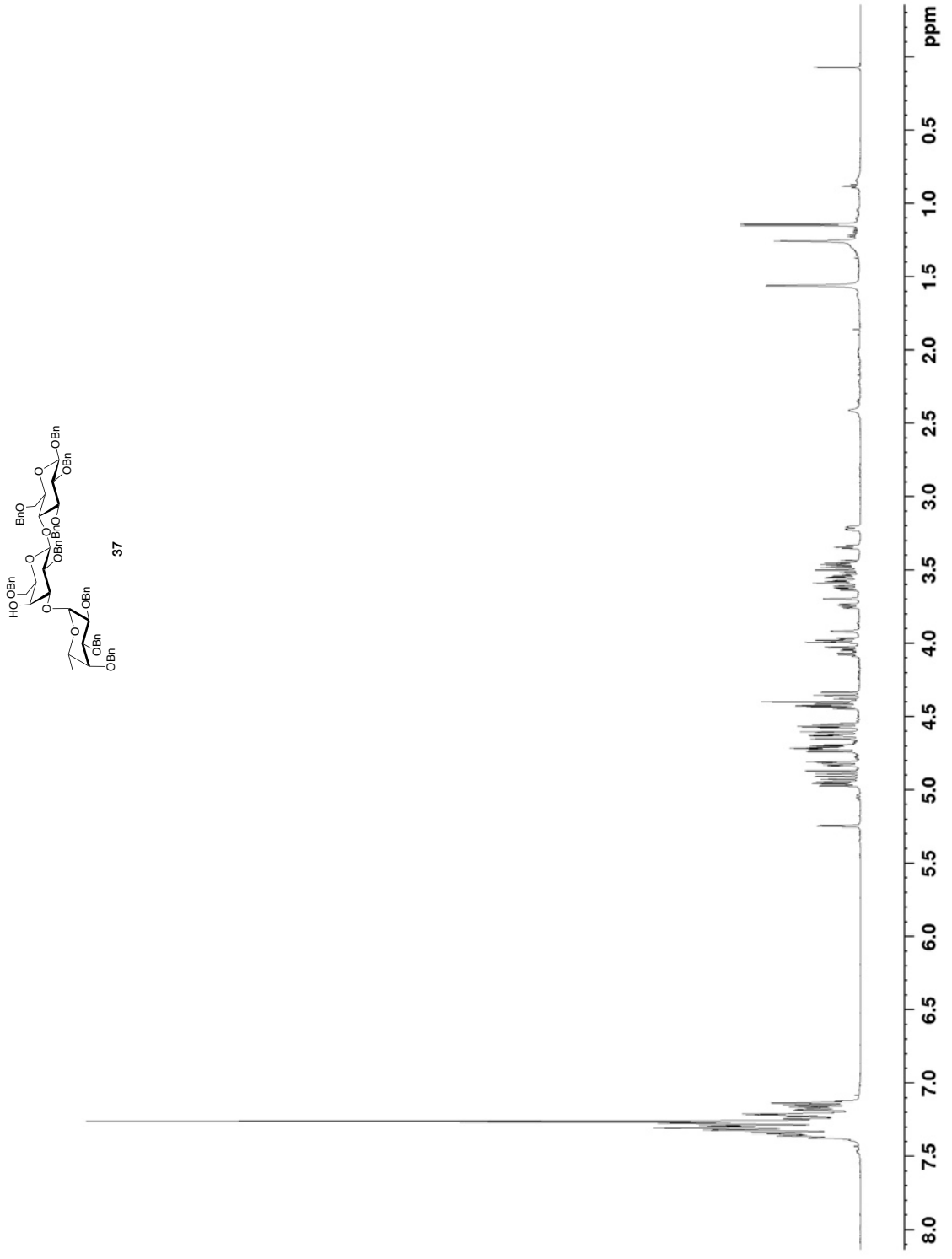


Figure A3. 45 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 37

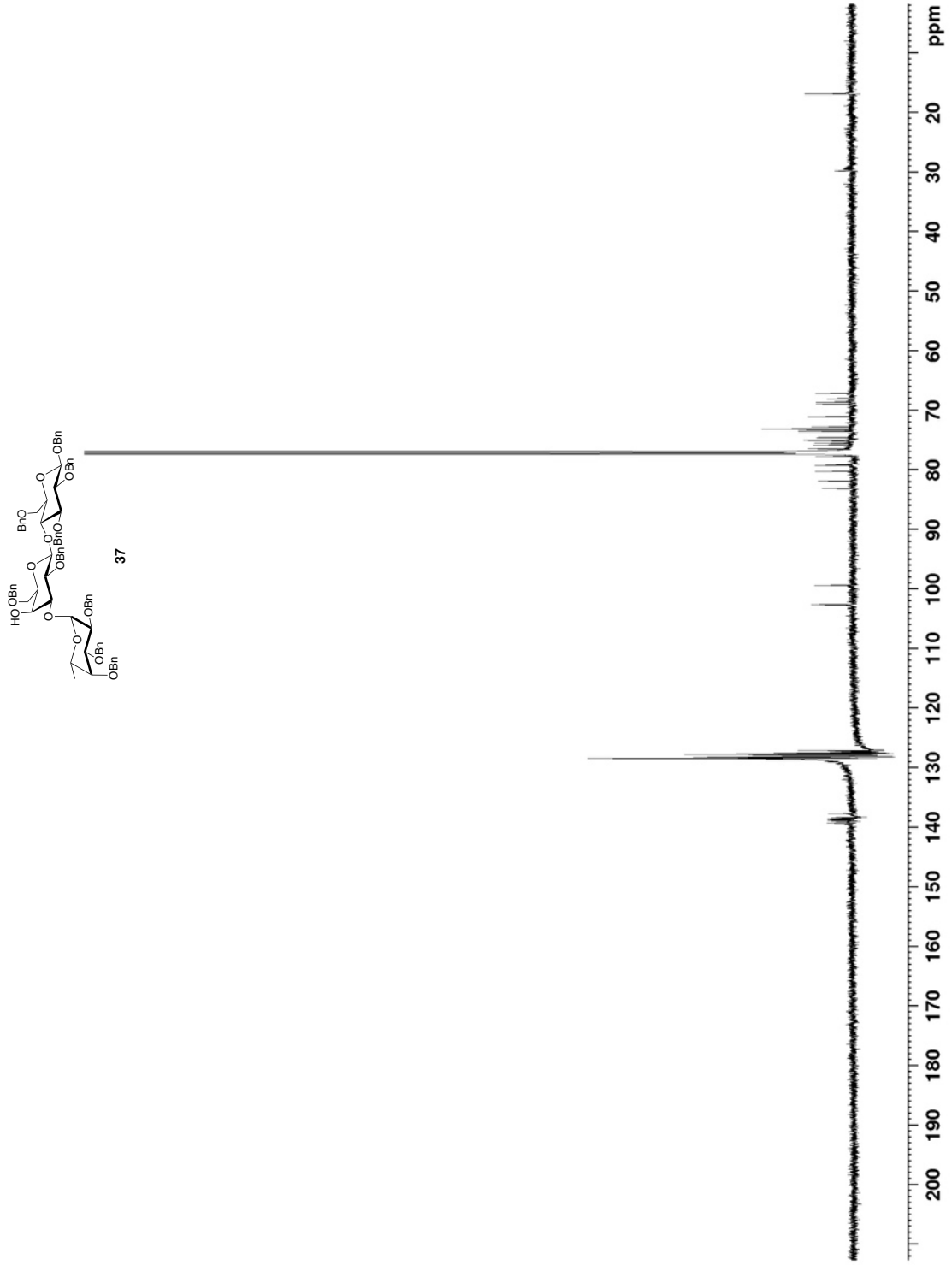


Figure A3. 46 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 37

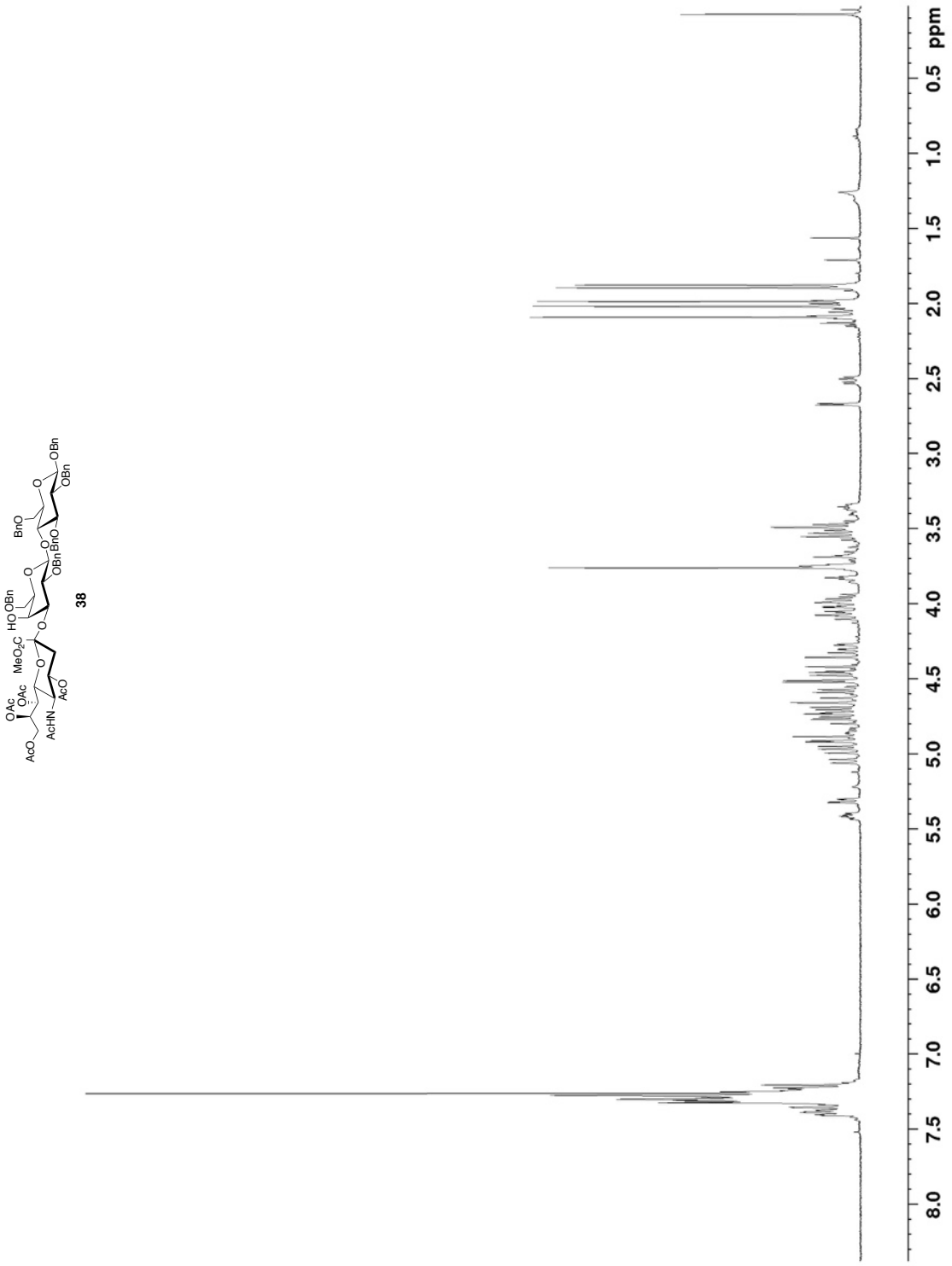


Figure A3. 47 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 38

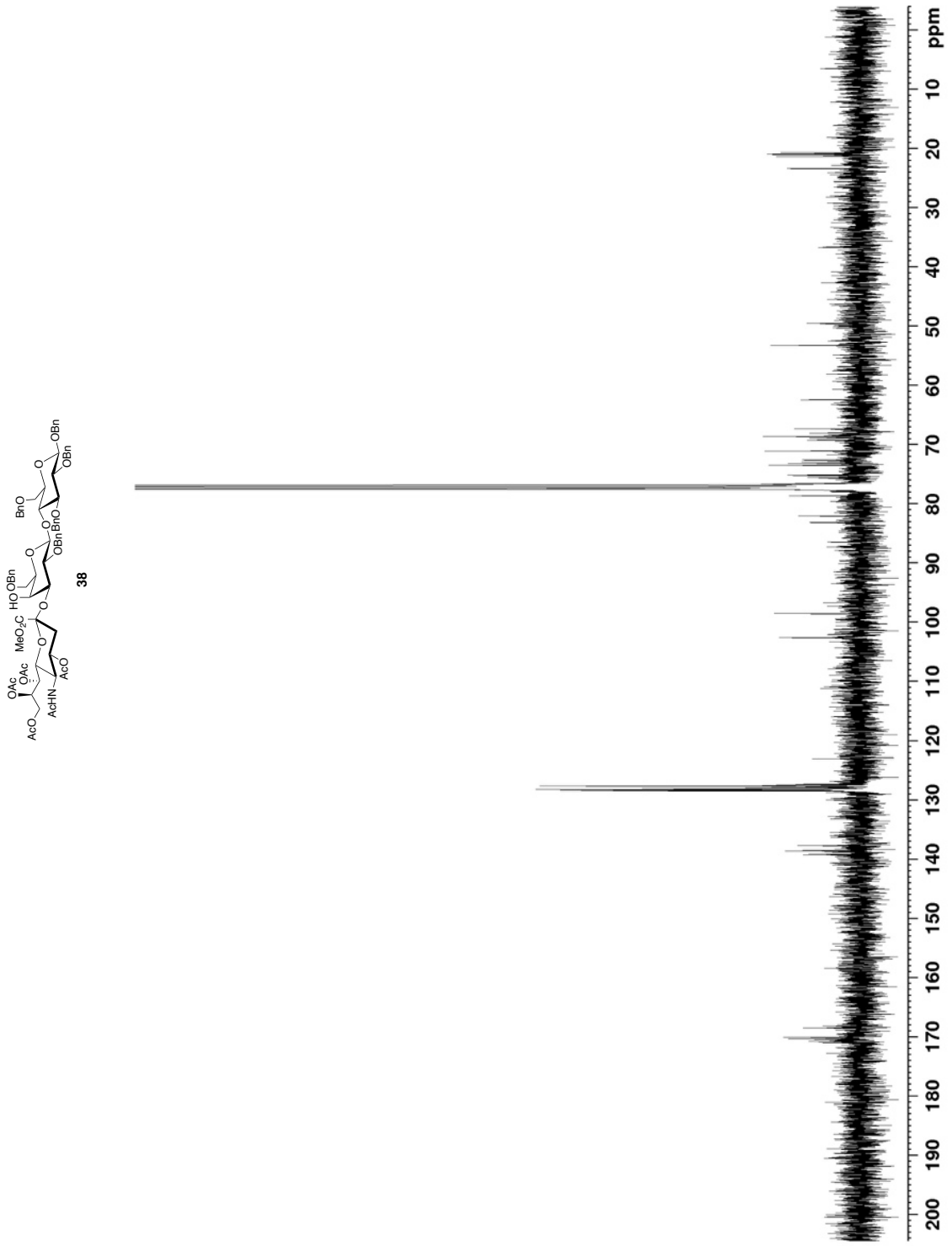


Figure A3. 48 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 38

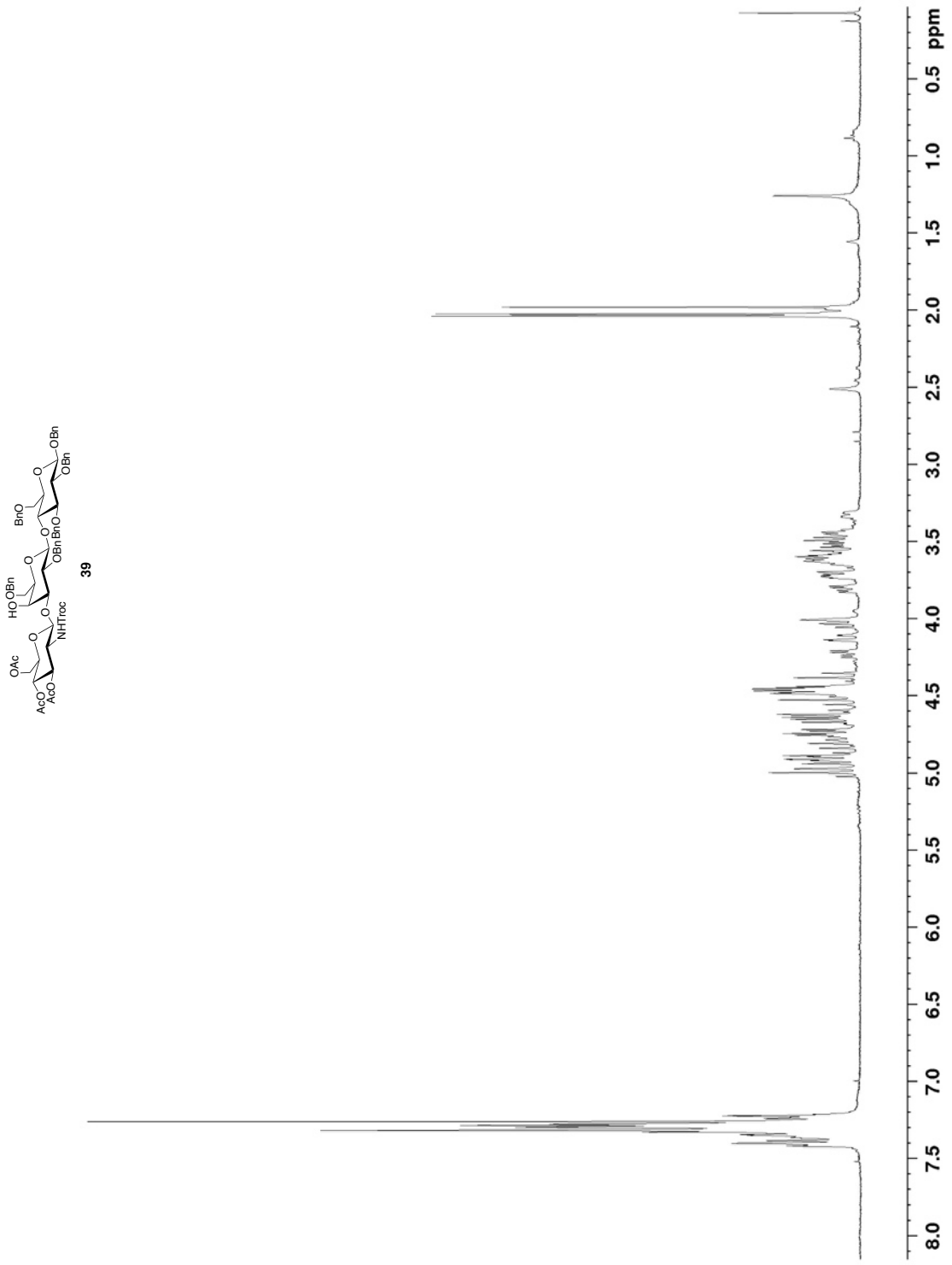


Figure A3. 49 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 39

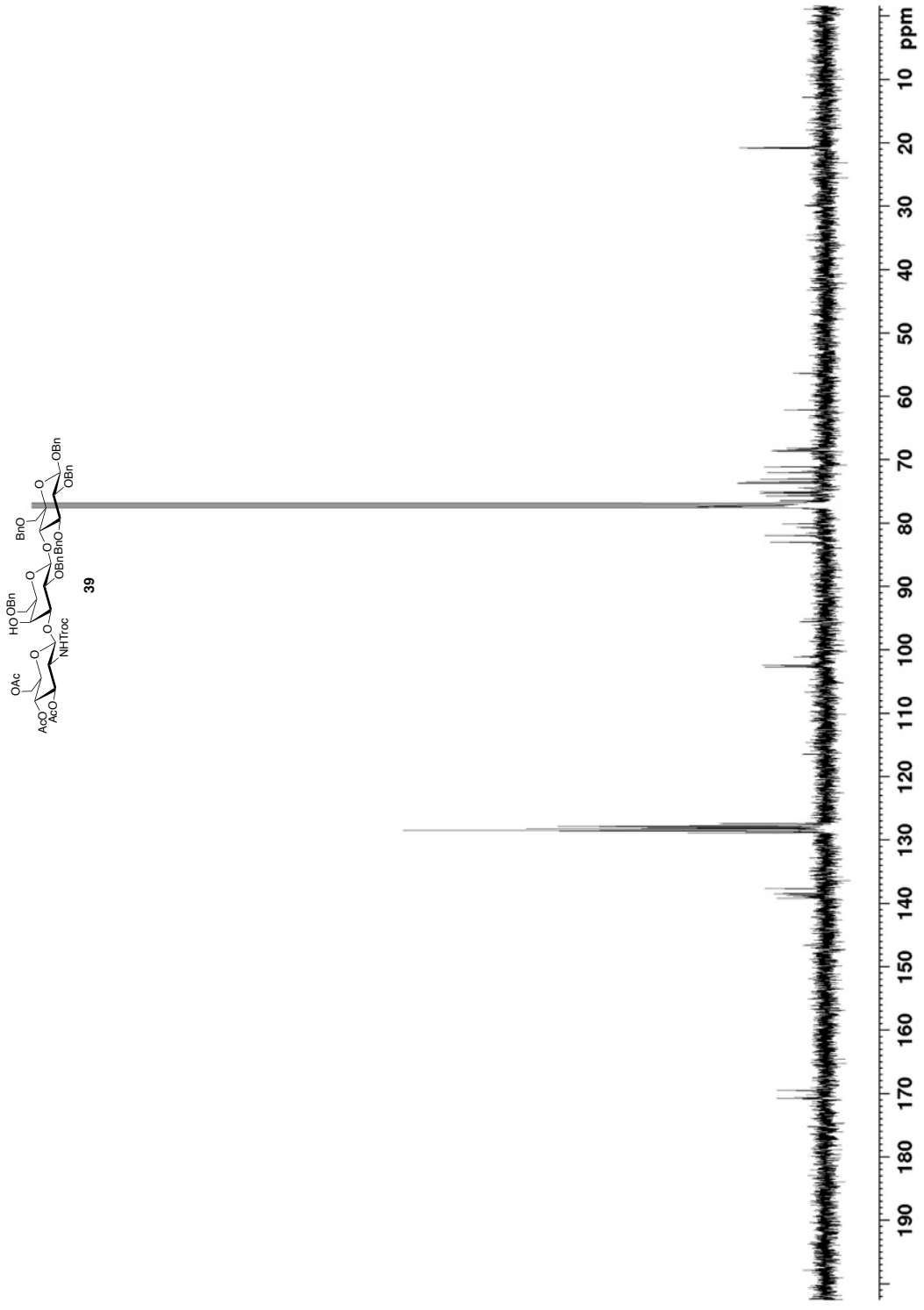


Figure A3. 50 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 39

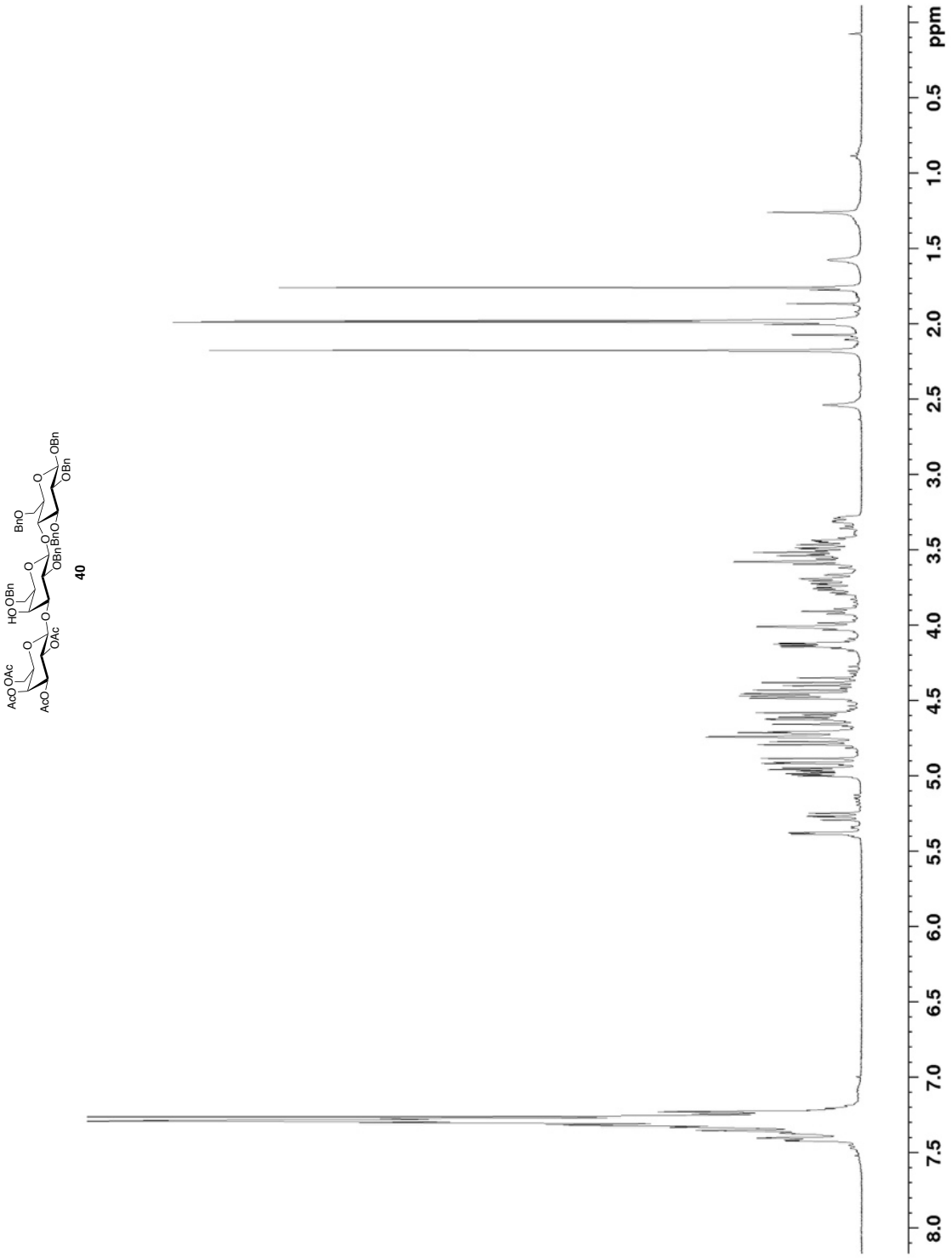


Figure A3. 51 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 40

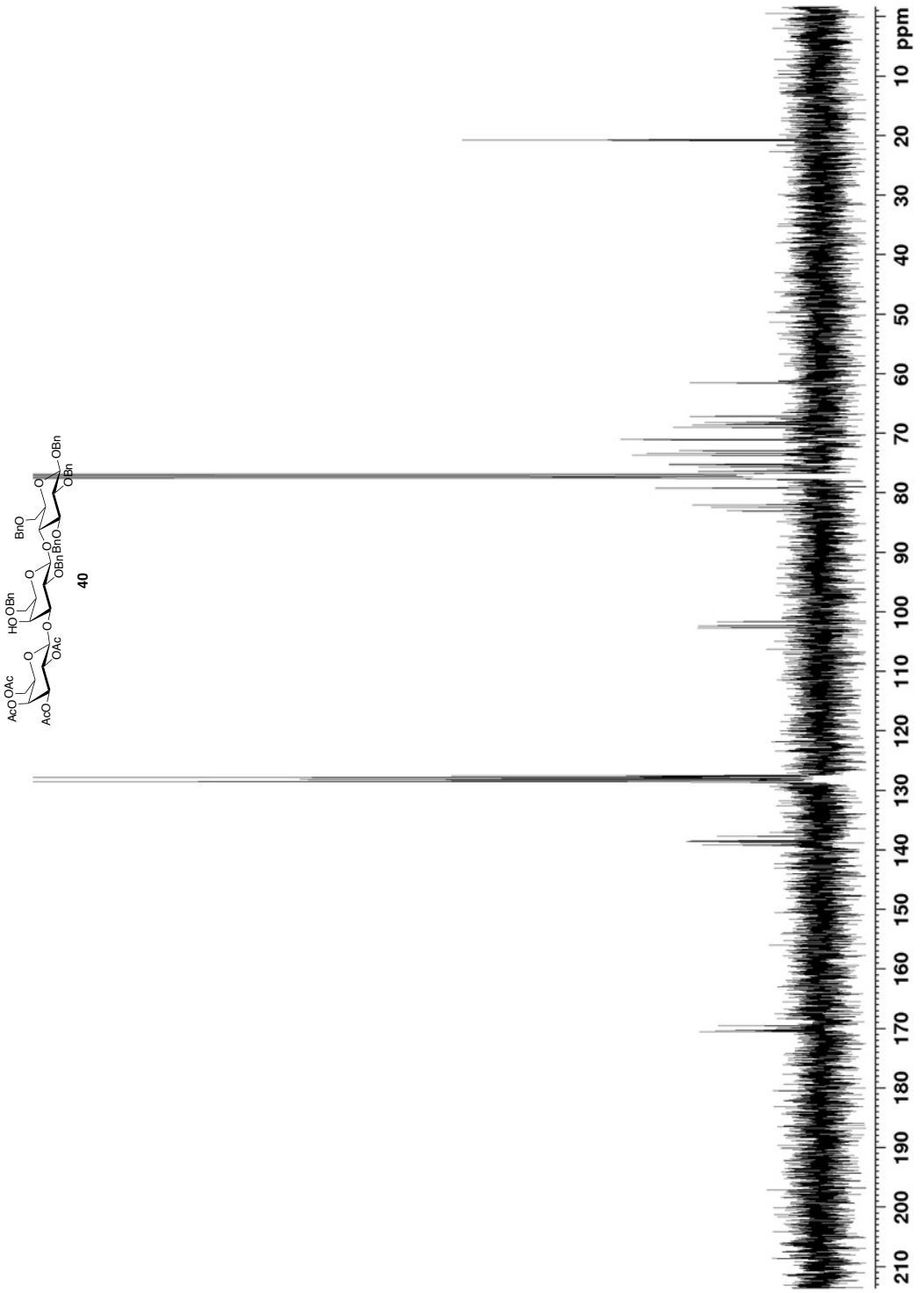


Figure A3. 52 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 40

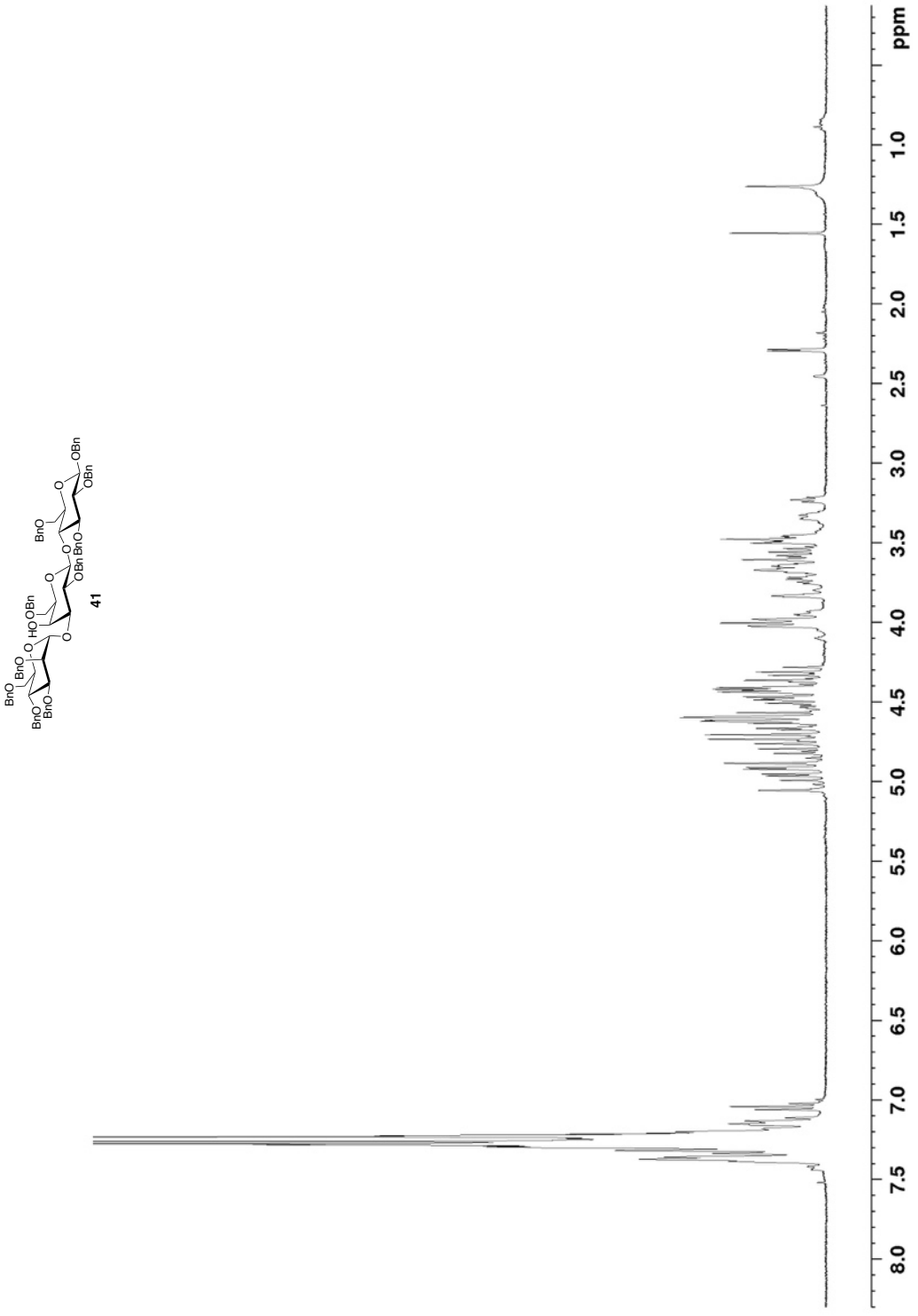


Figure A3. 53 ^{1}H NMR spectra (600 MHz, $CDCl_3$) of compound 41

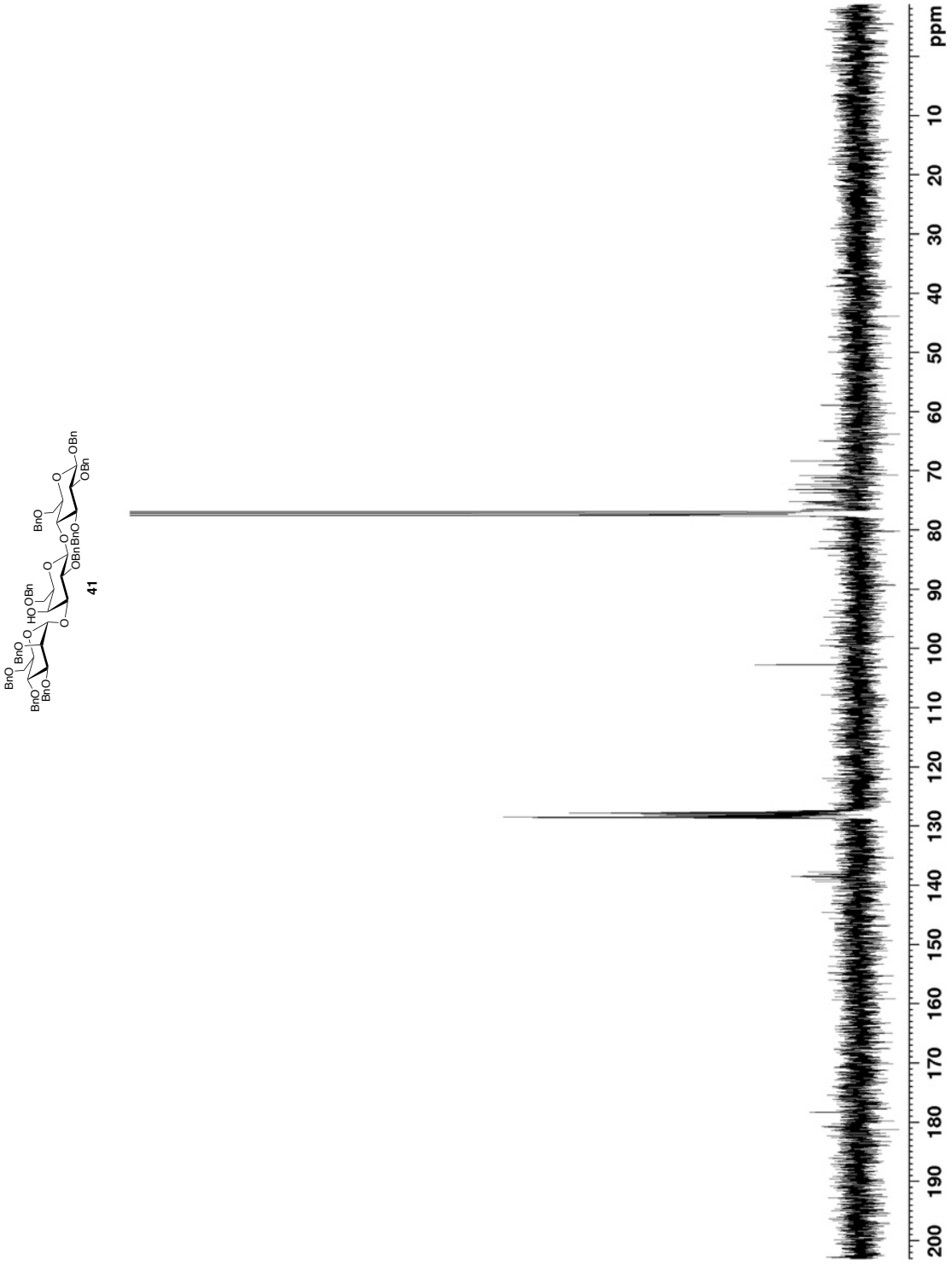


Figure A3. 54 ^{13}C NMR spectra (150 MHz, $CDCl_3$) of compound 41

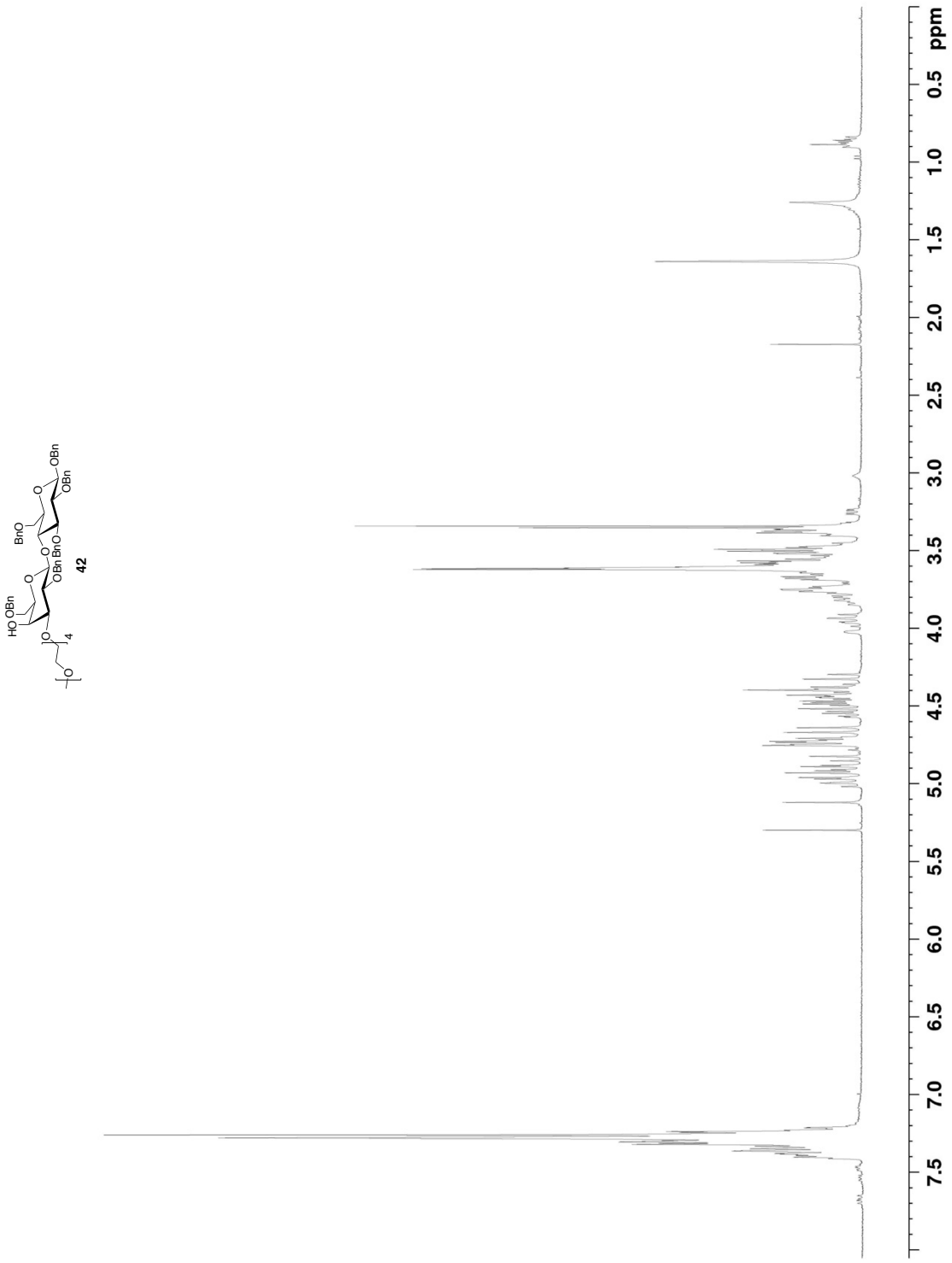


Figure A3. 55 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 42

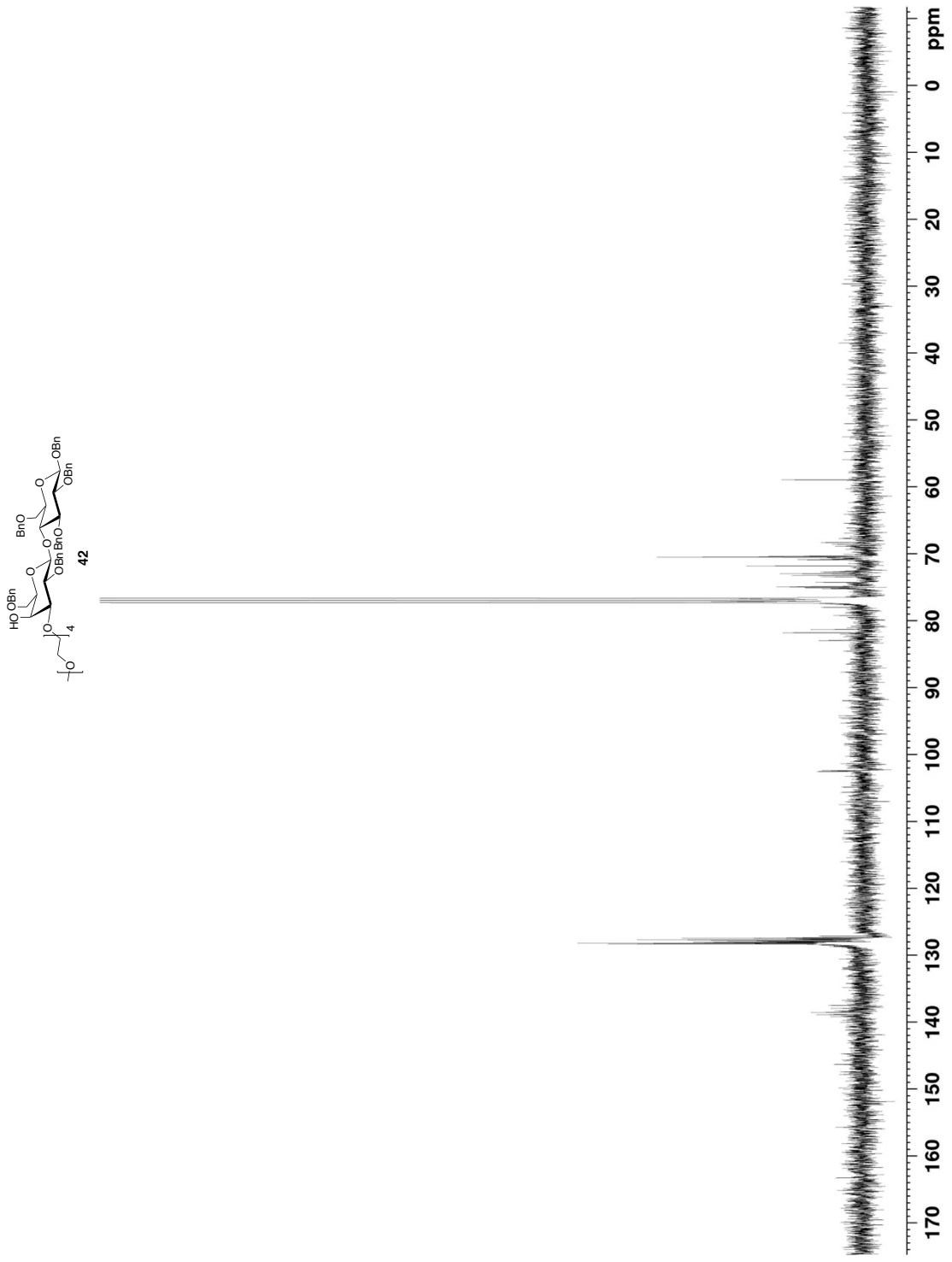


Figure A3. 56 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 42

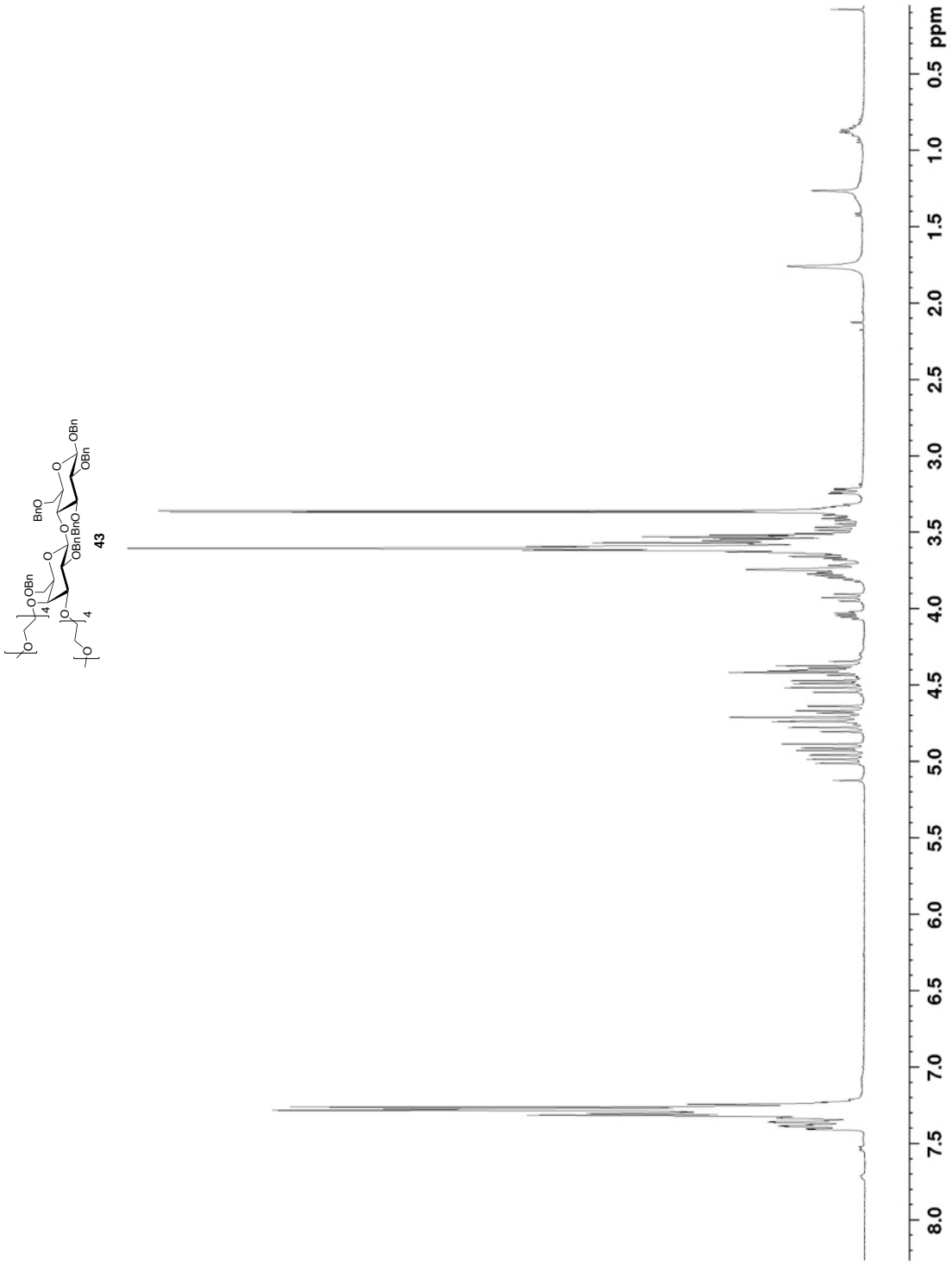


Figure A3. 57 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 43

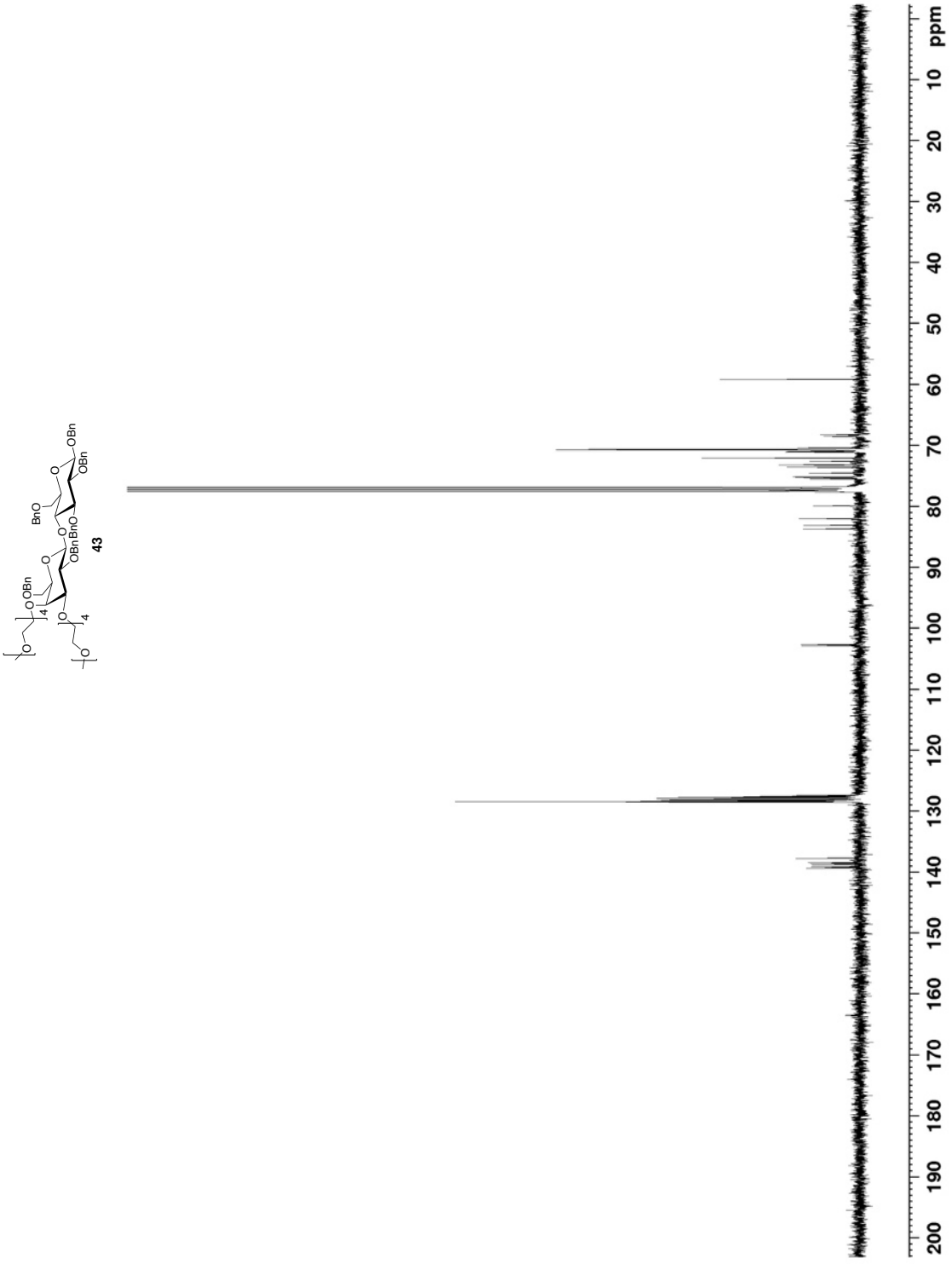


Figure A3. 58 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 43

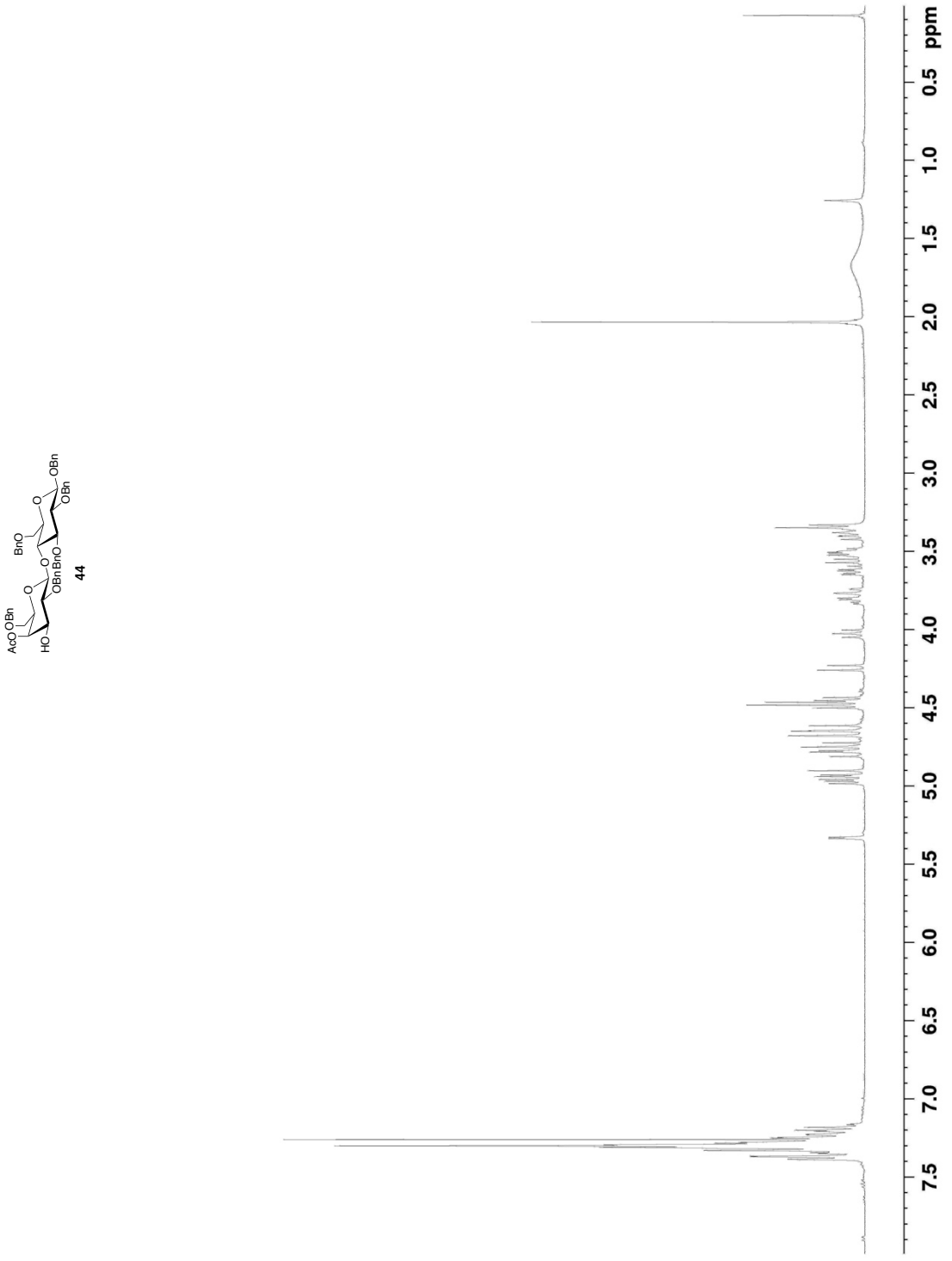


Figure A3. 59 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 44

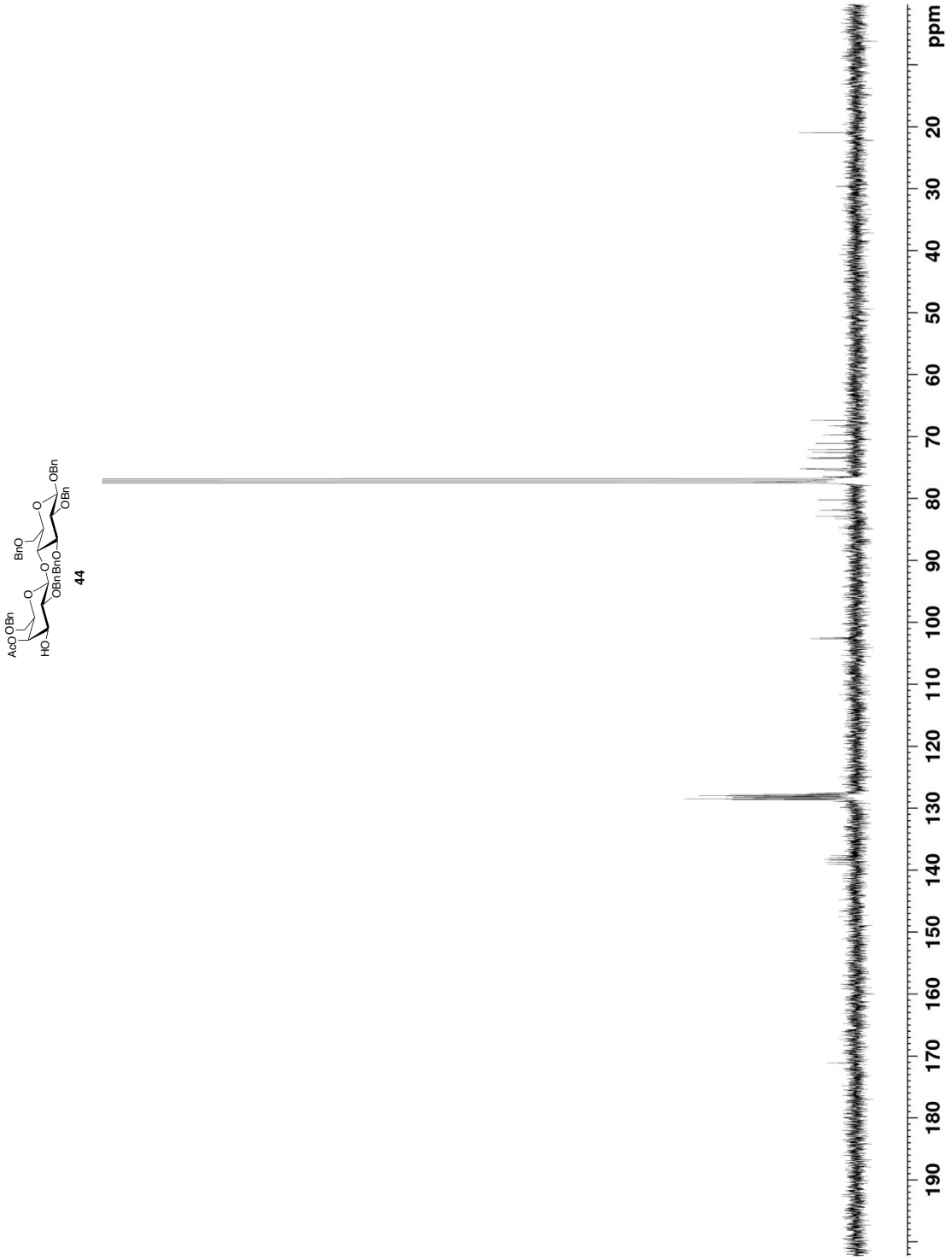


Figure A3. 60 ^{31}C NMR spectra (100 MHz, $CDCl_3$) of compound 44

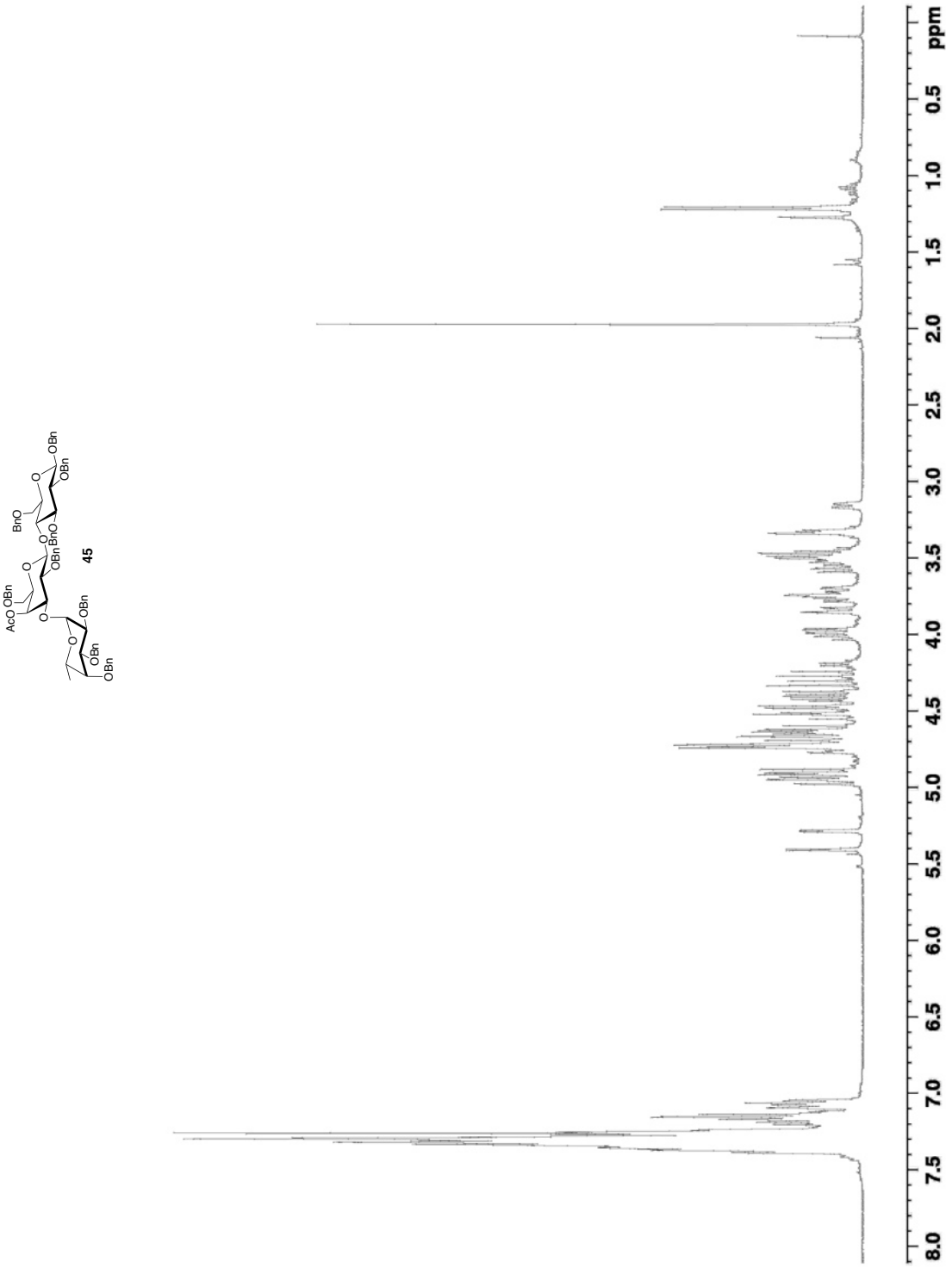


Figure A3. 61 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 45

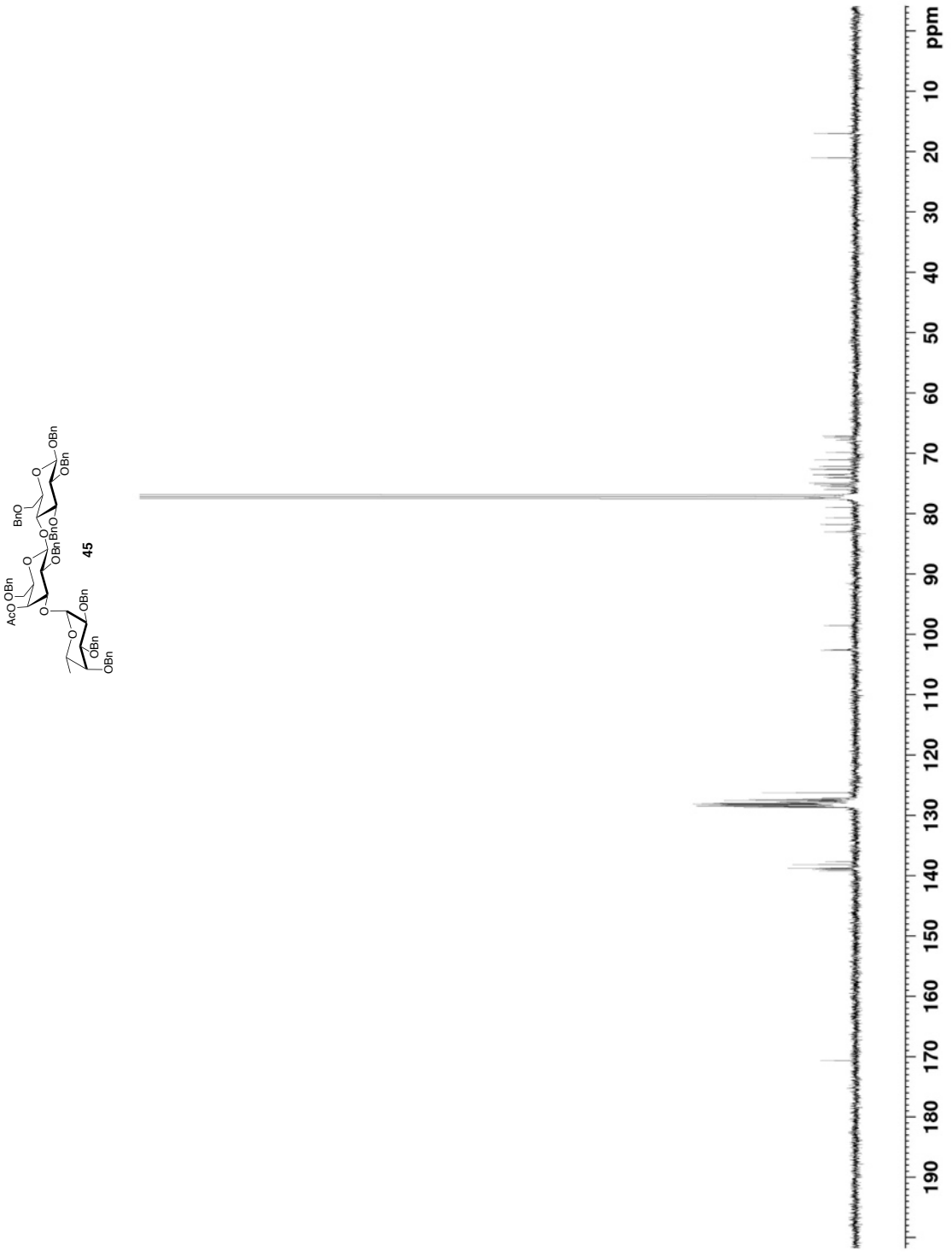


Figure A3. 62 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 45

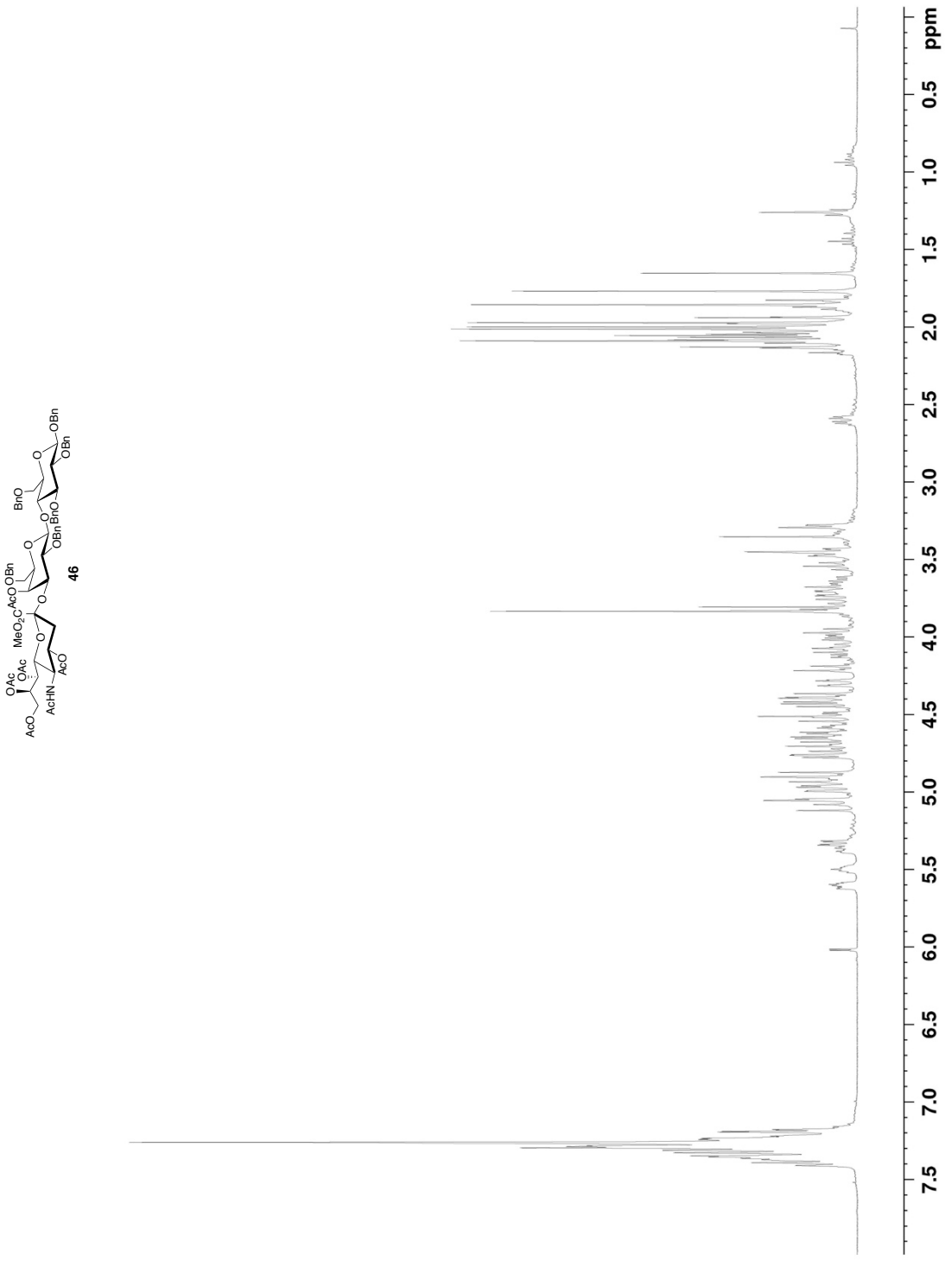


Figure A3. 63 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 46

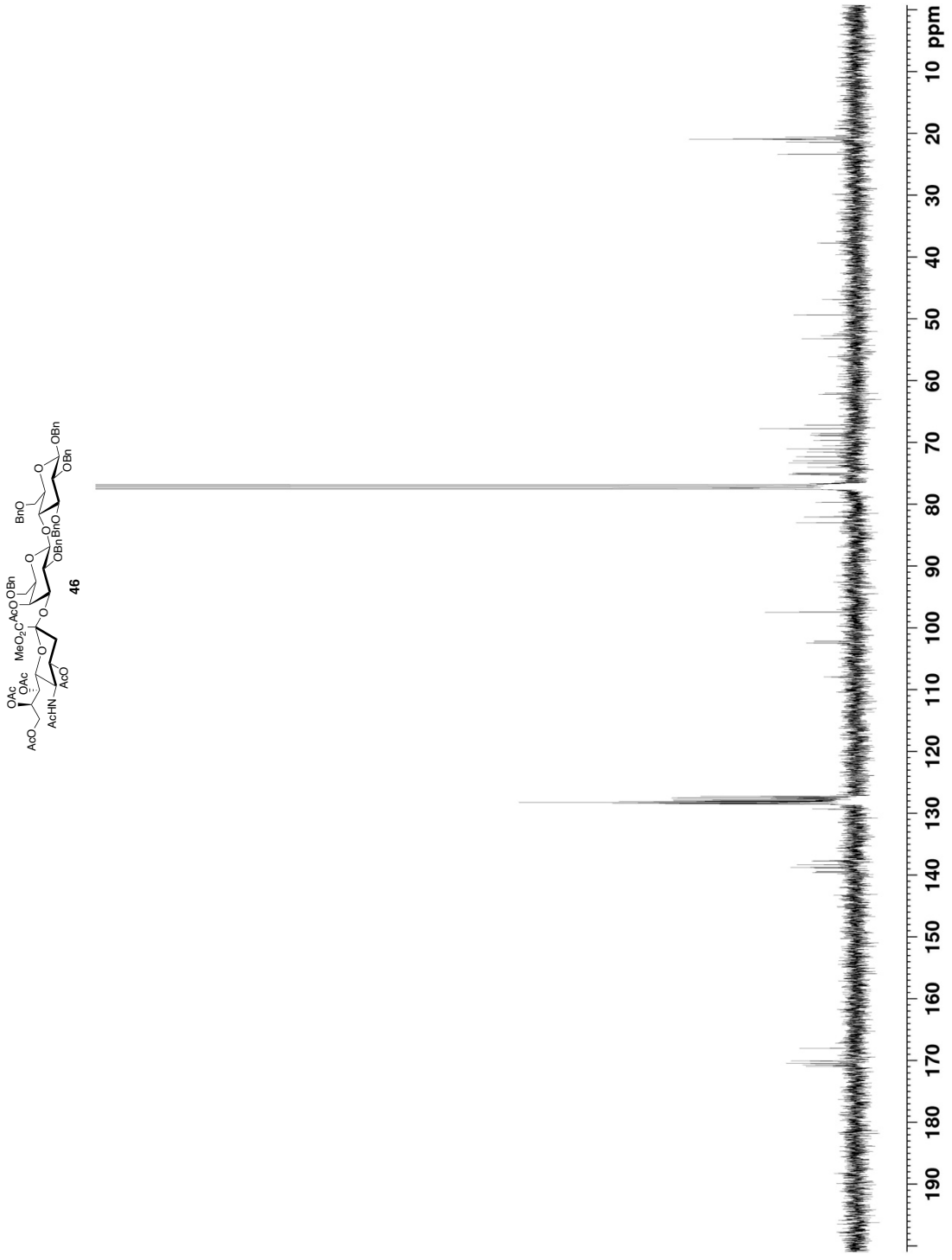


Figure A3. 64 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 46

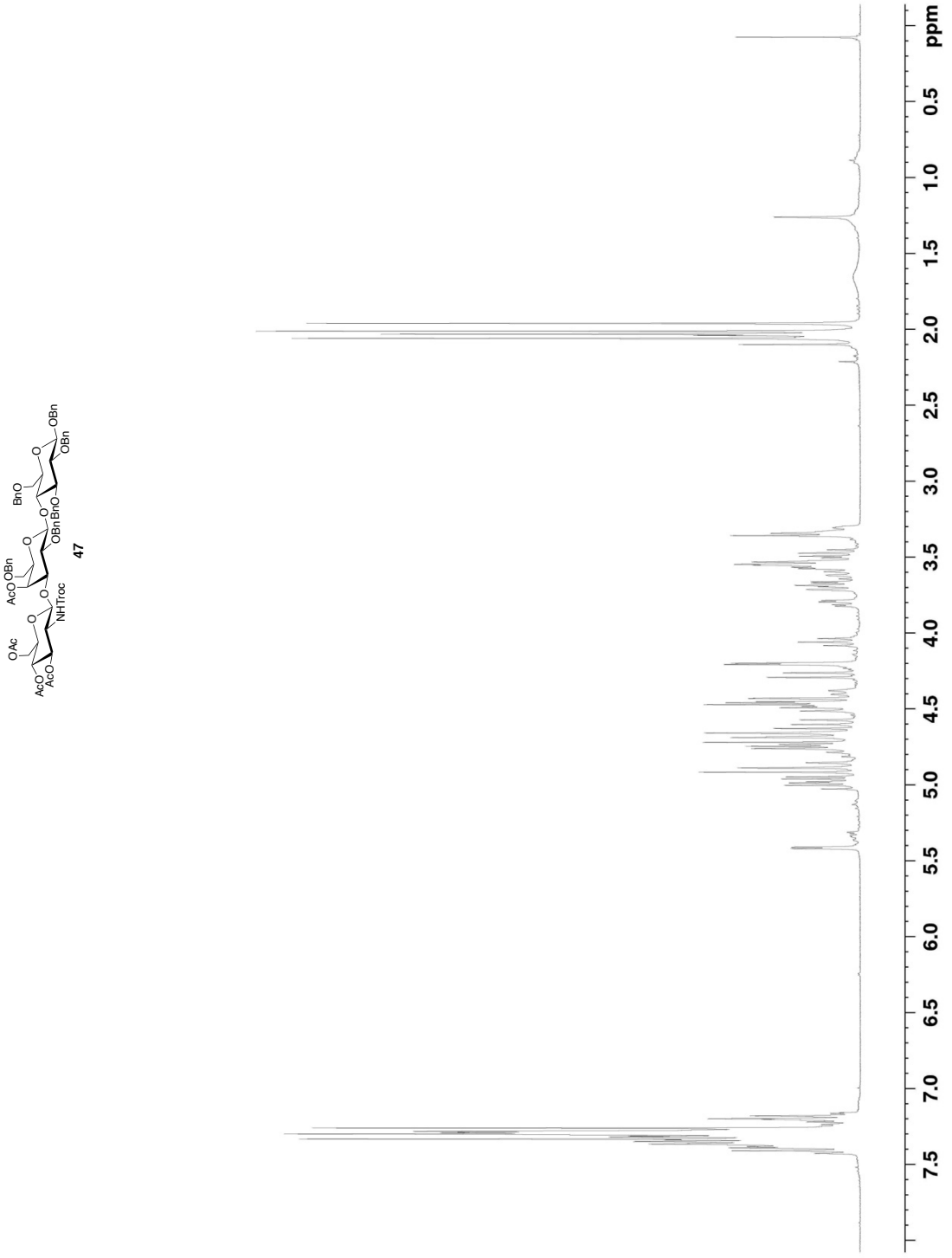


Figure A3. 65 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 47

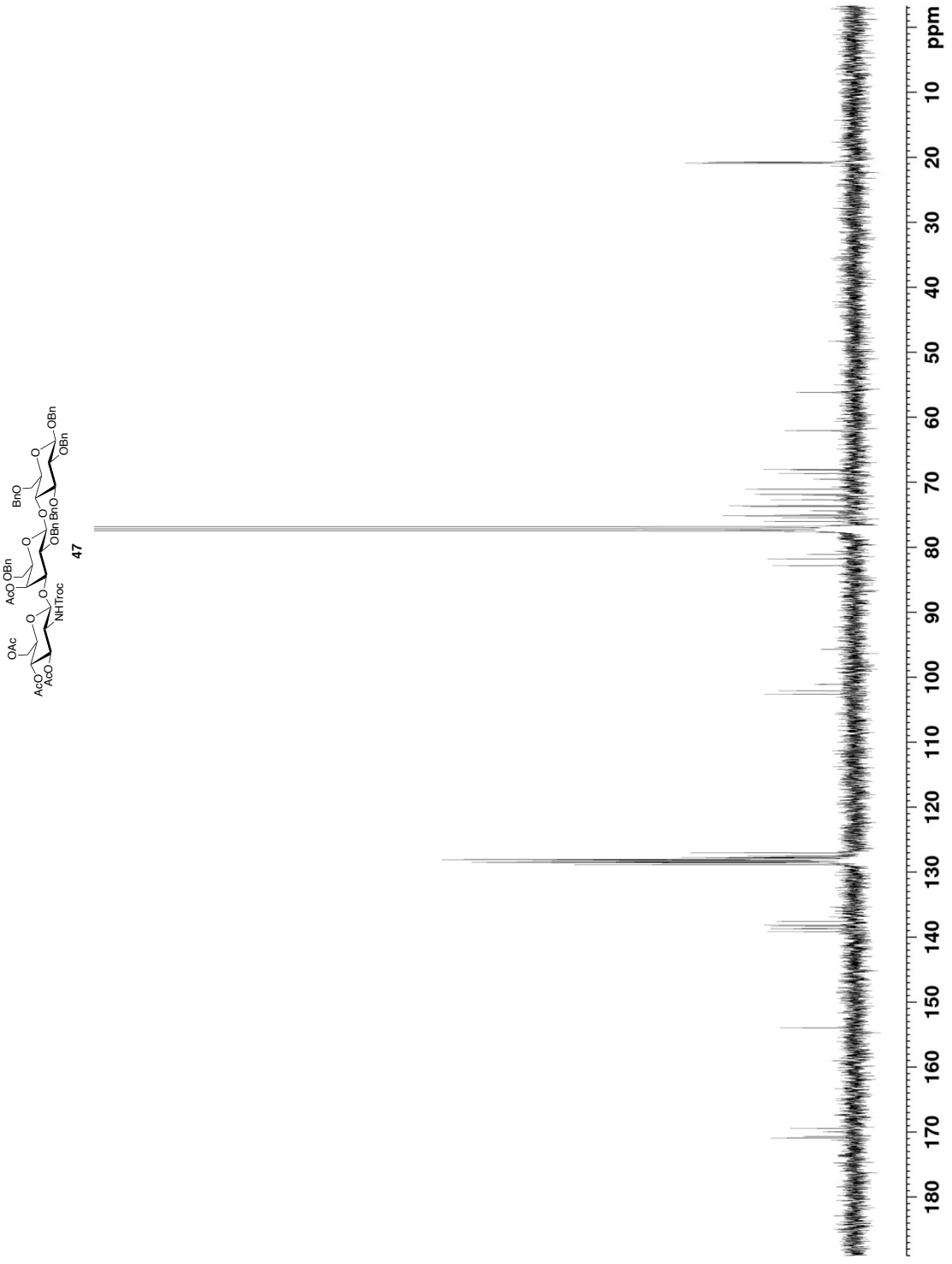


Figure A3. 66 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 47

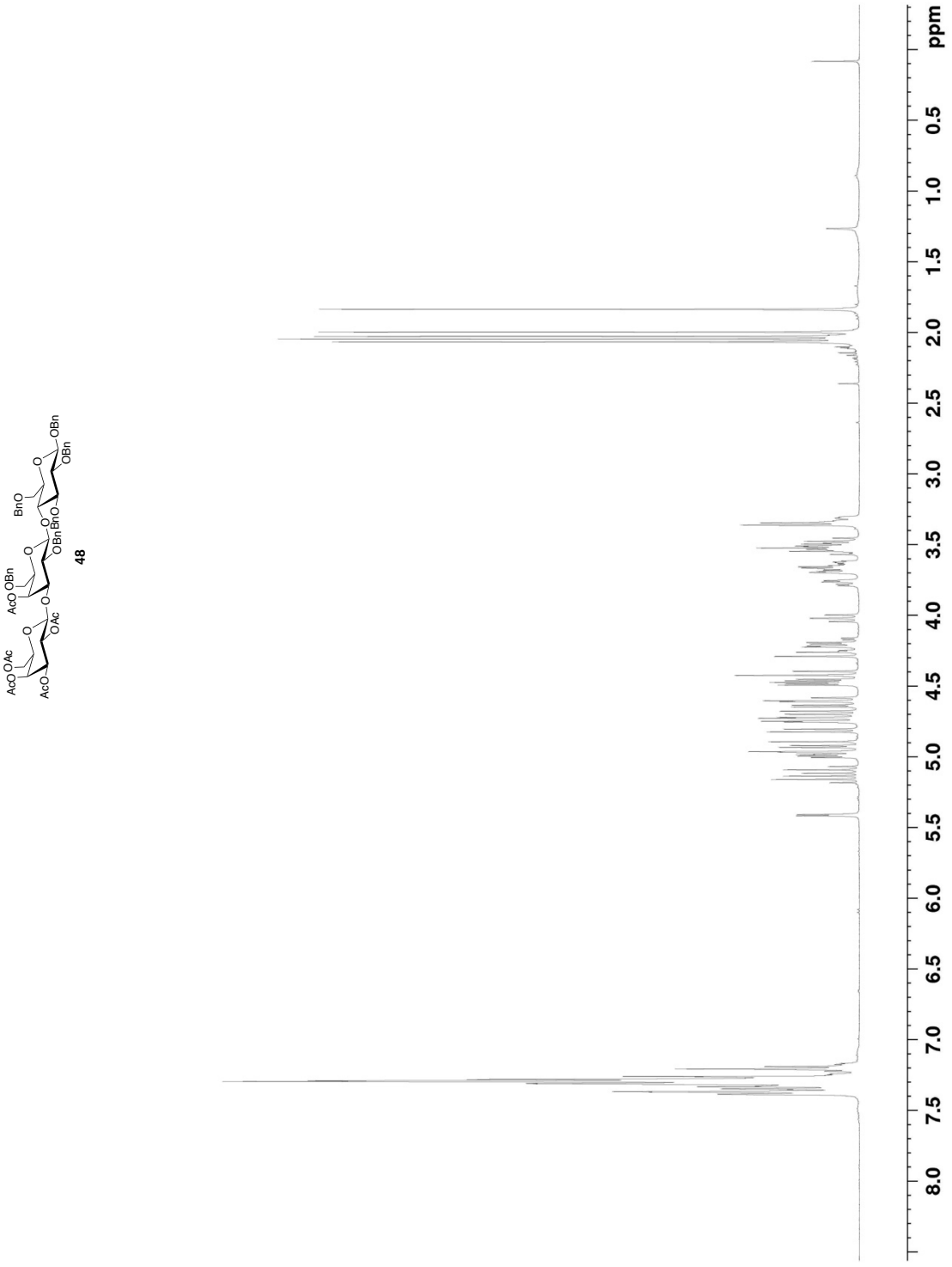


Figure A3. 67 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 48

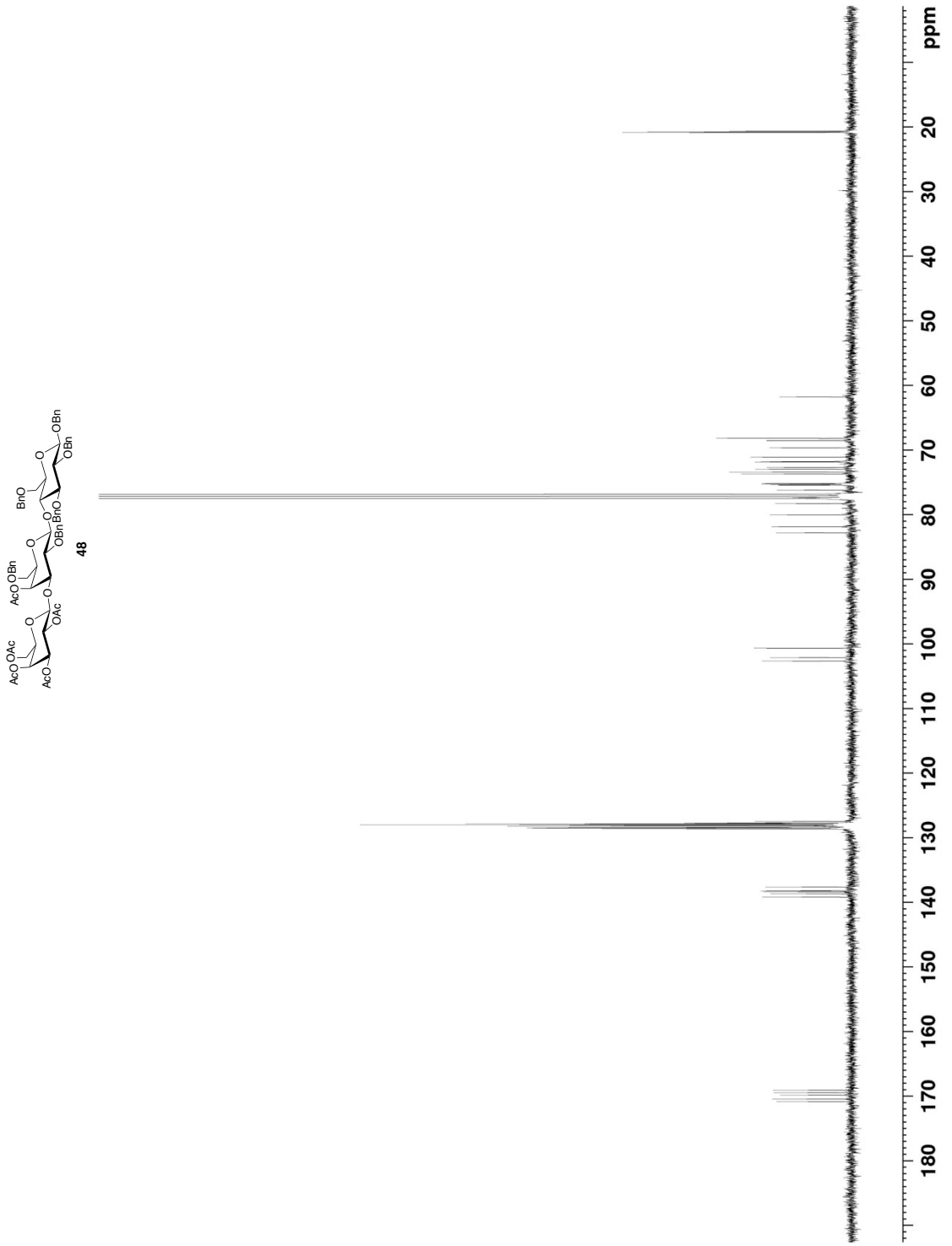


Figure A3. 68 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 48

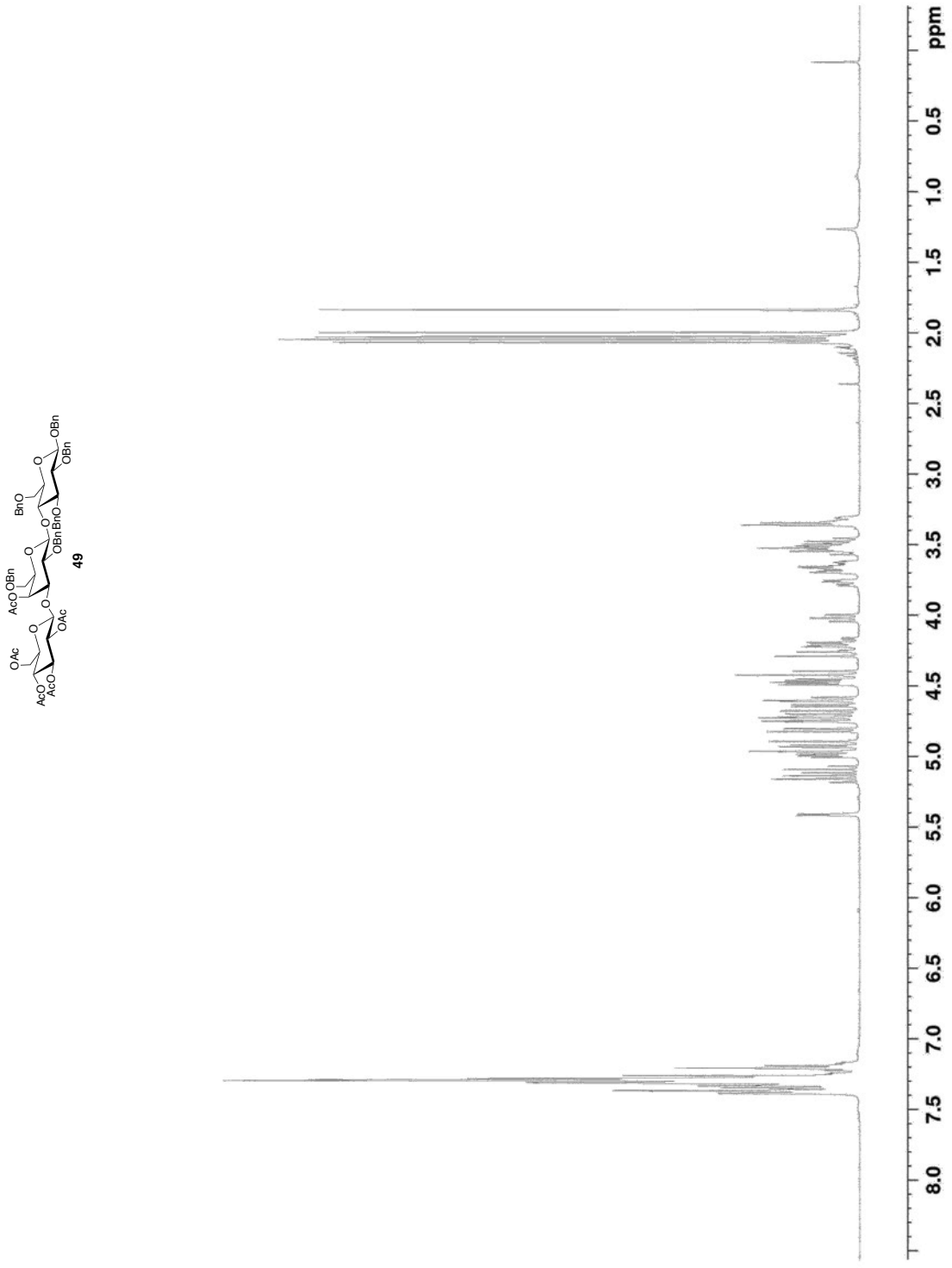


Figure A3. 69 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 49

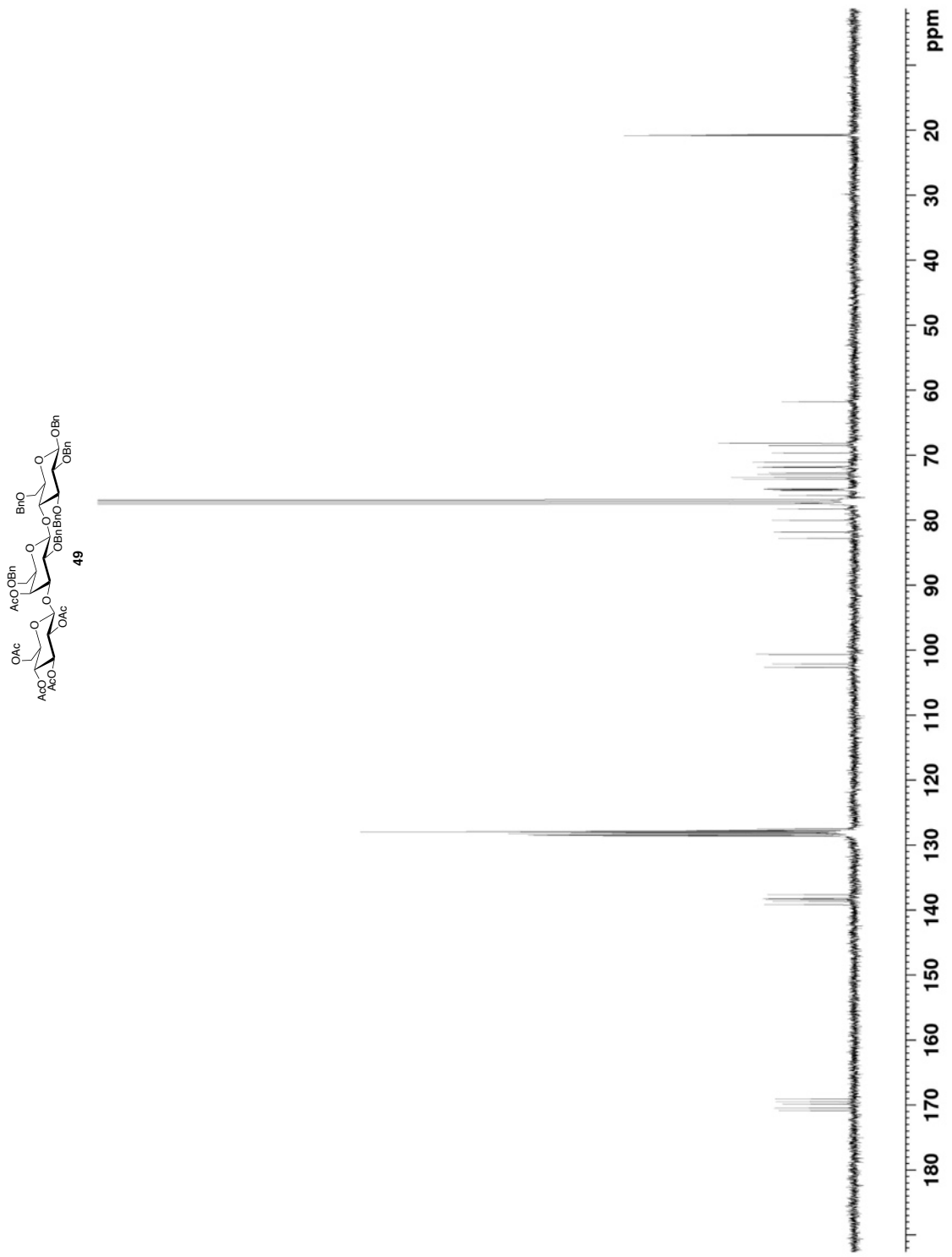


Figure A3. 70 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 49

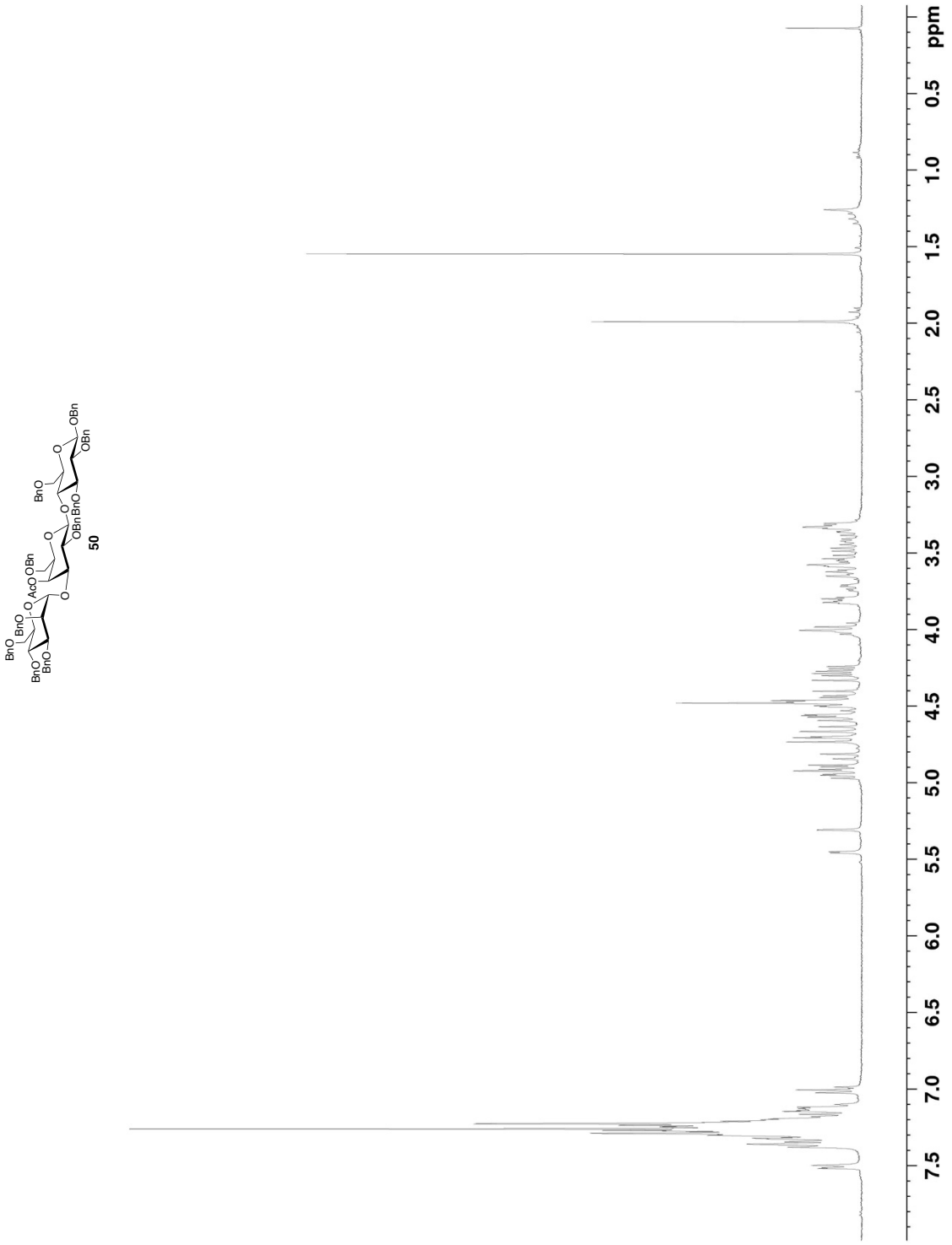


Figure A3. 71 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 50

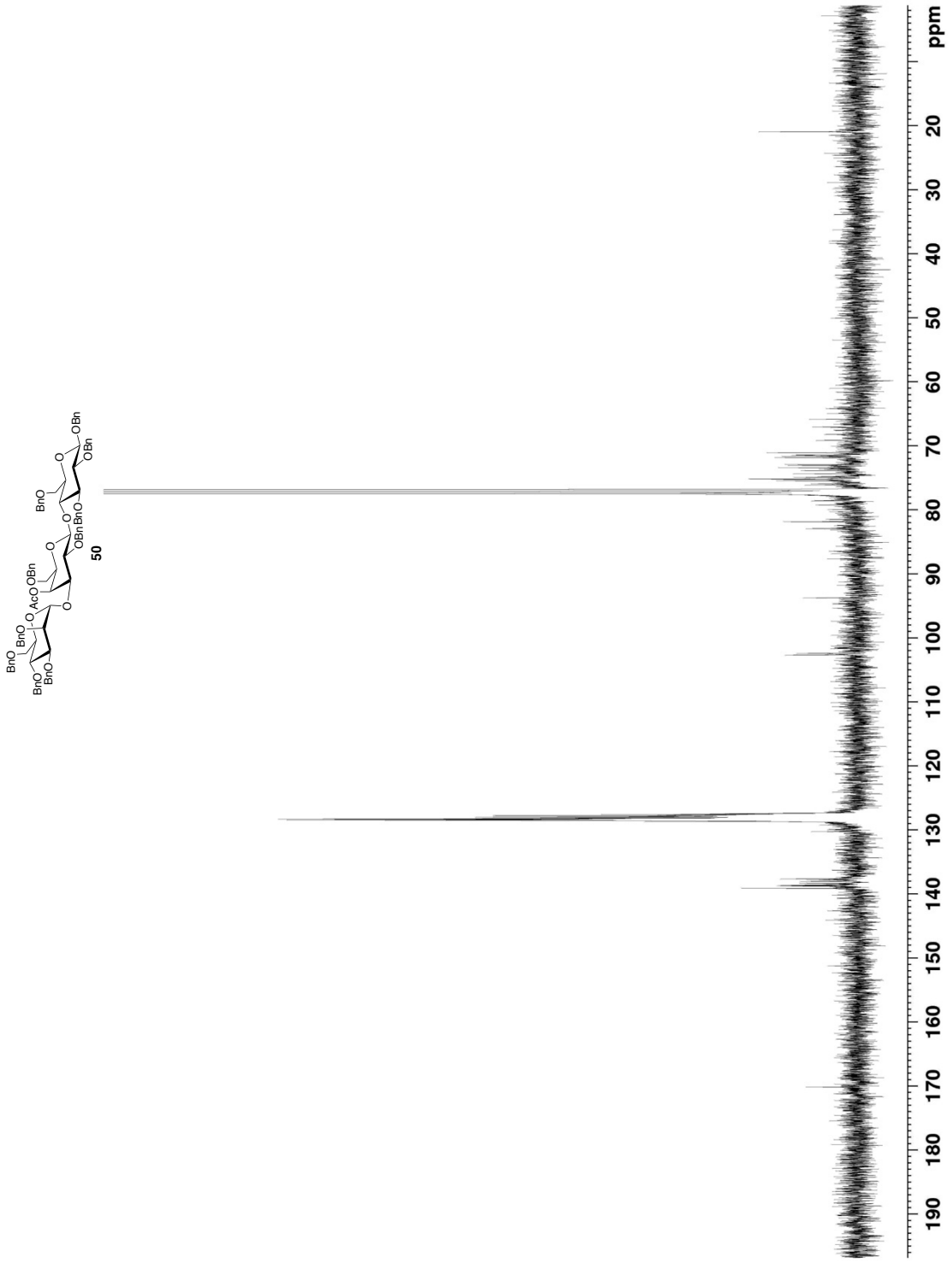


Figure A3. 72 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 50

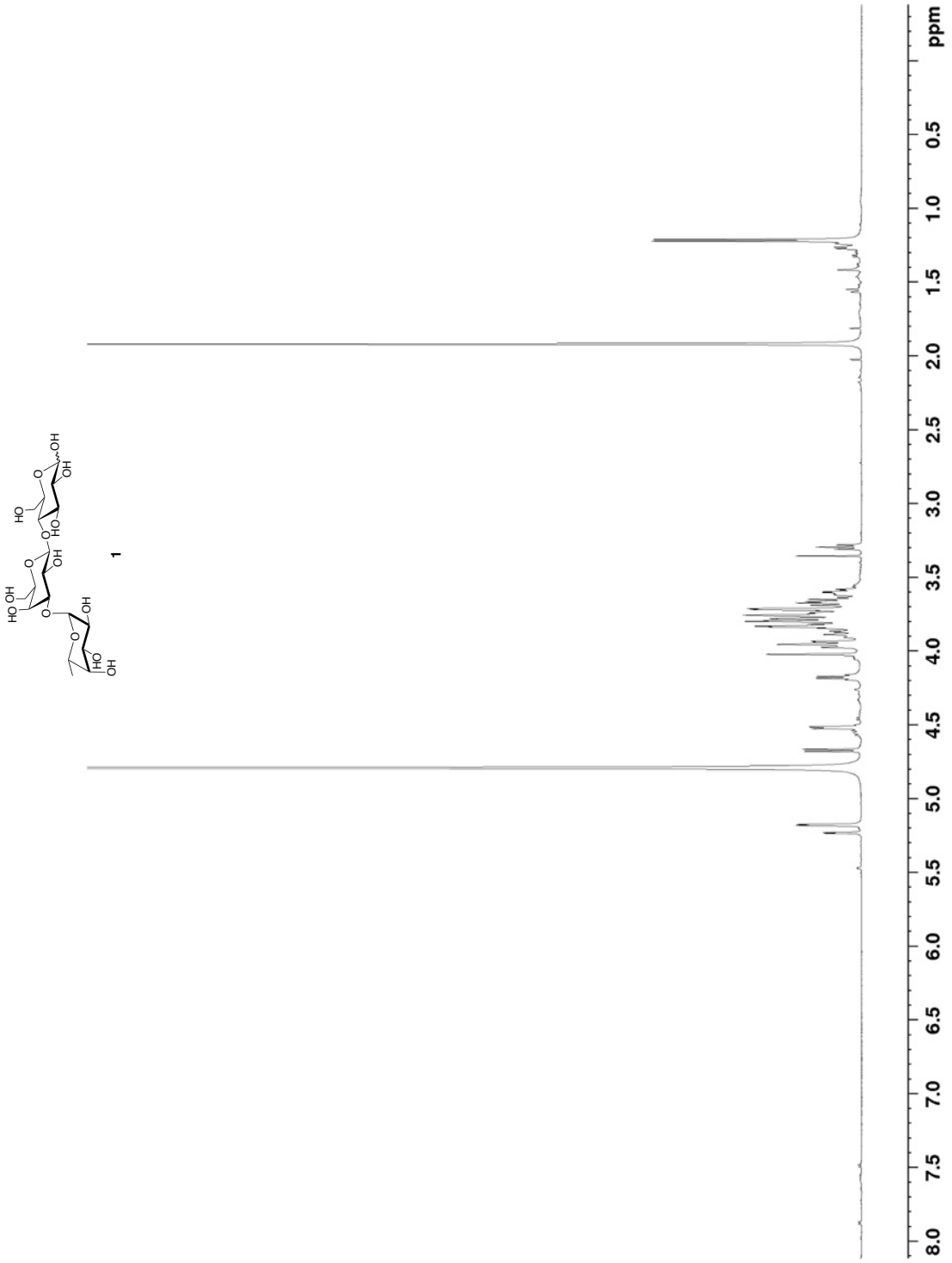


Figure A3. 73 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 1

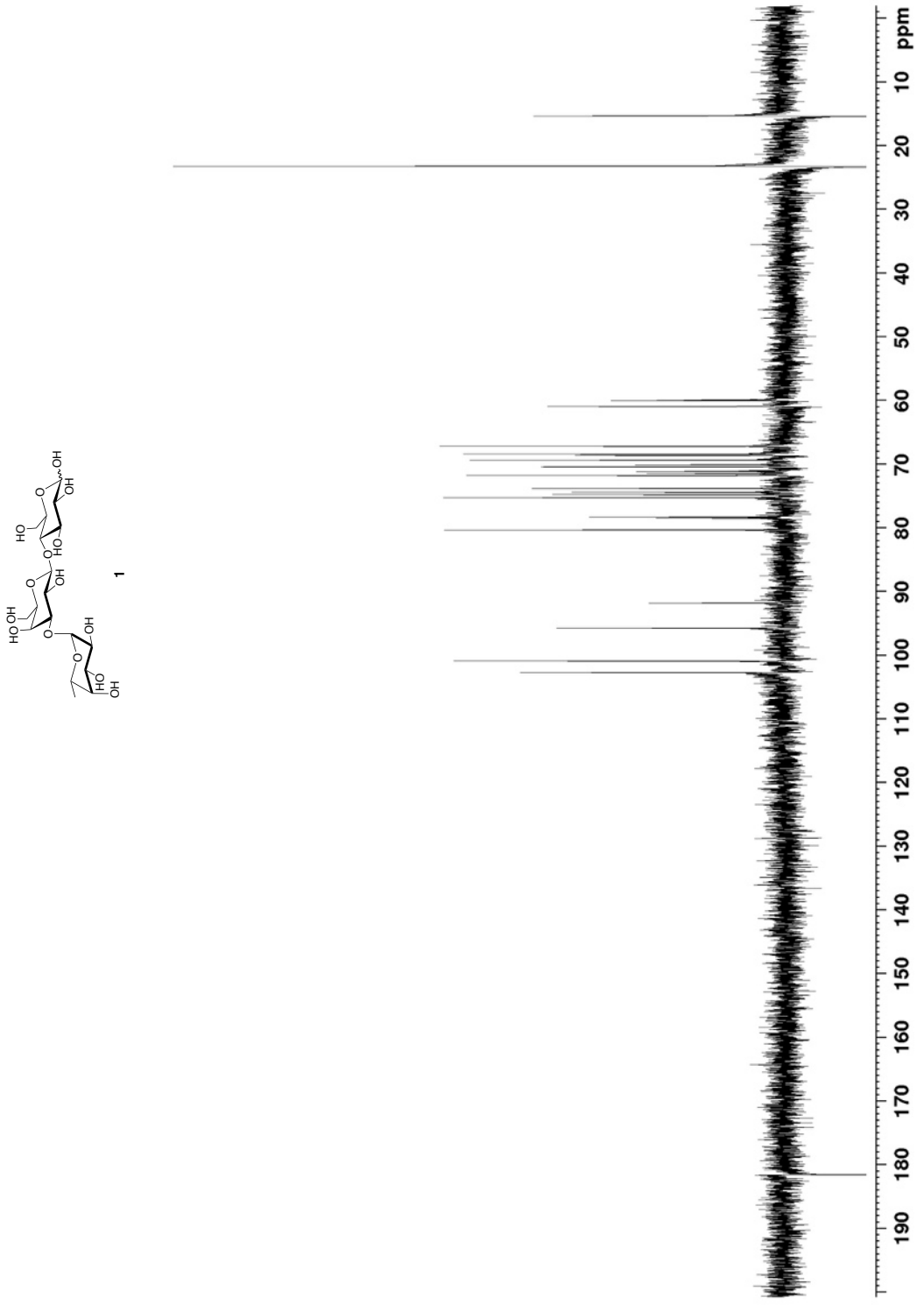


Figure A3. 74 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 1

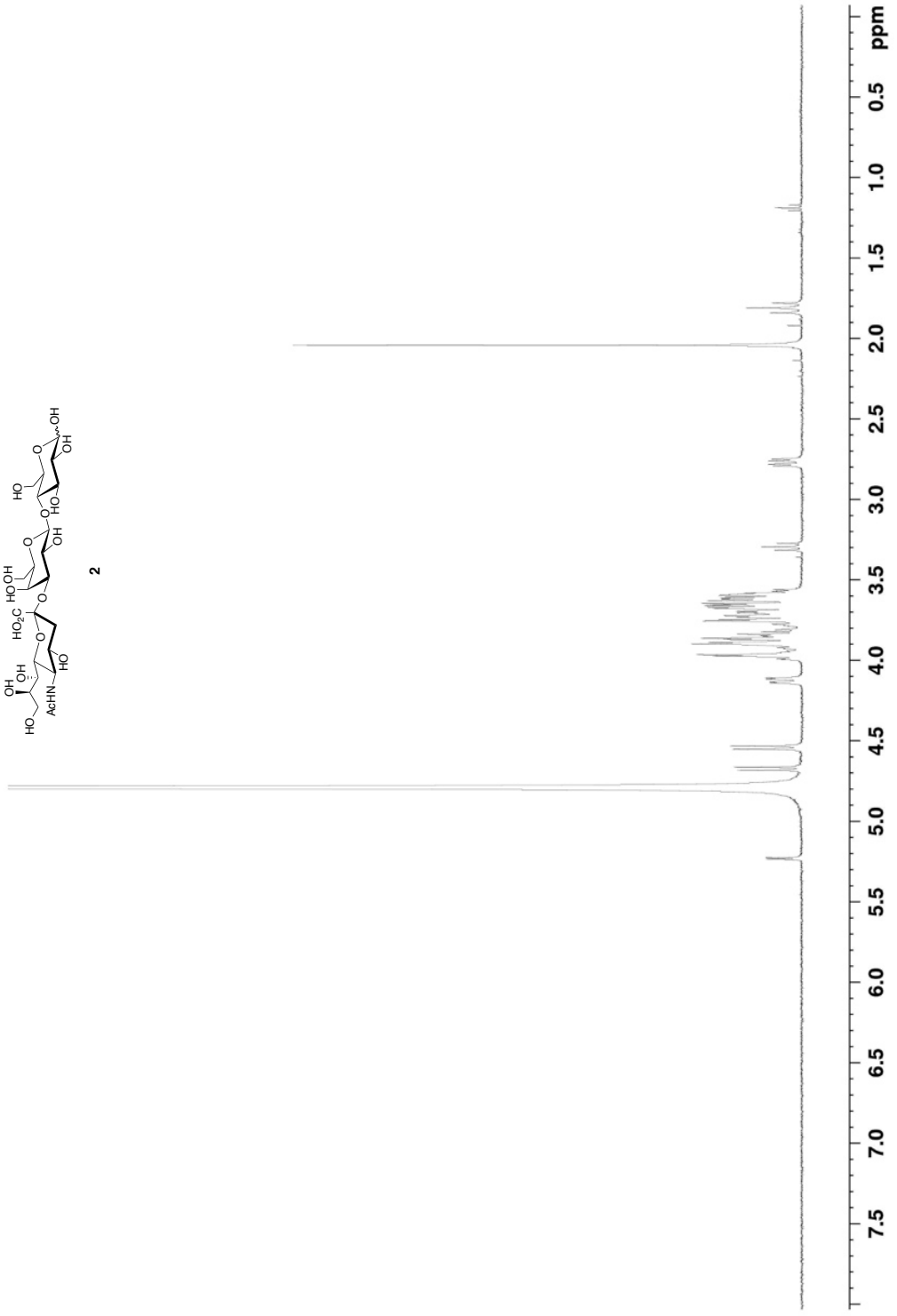


Figure A3. 75 1H NMR spectra (400 MHz, CDCl3) of compound 2

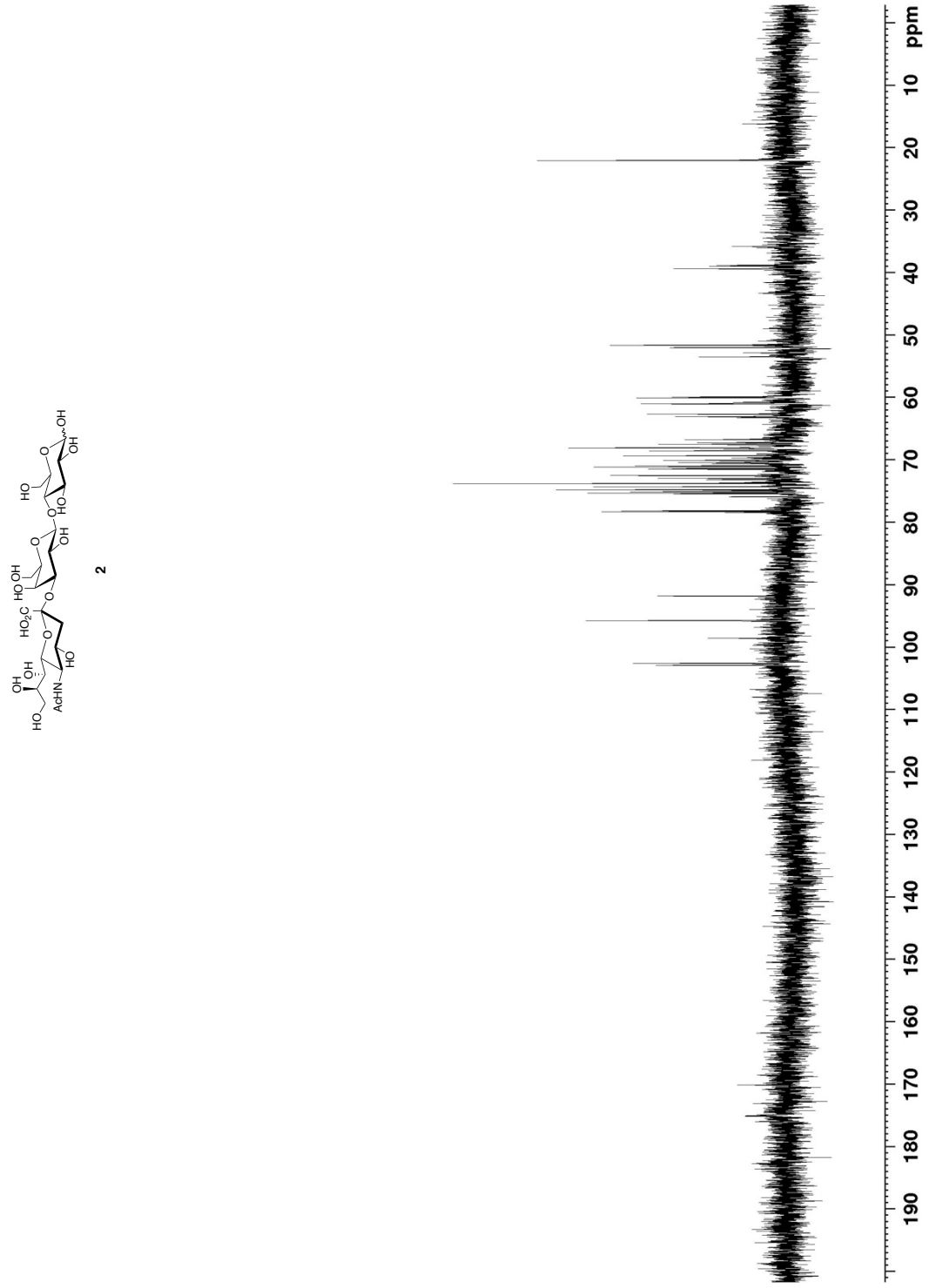


Figure A3. 76 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 2

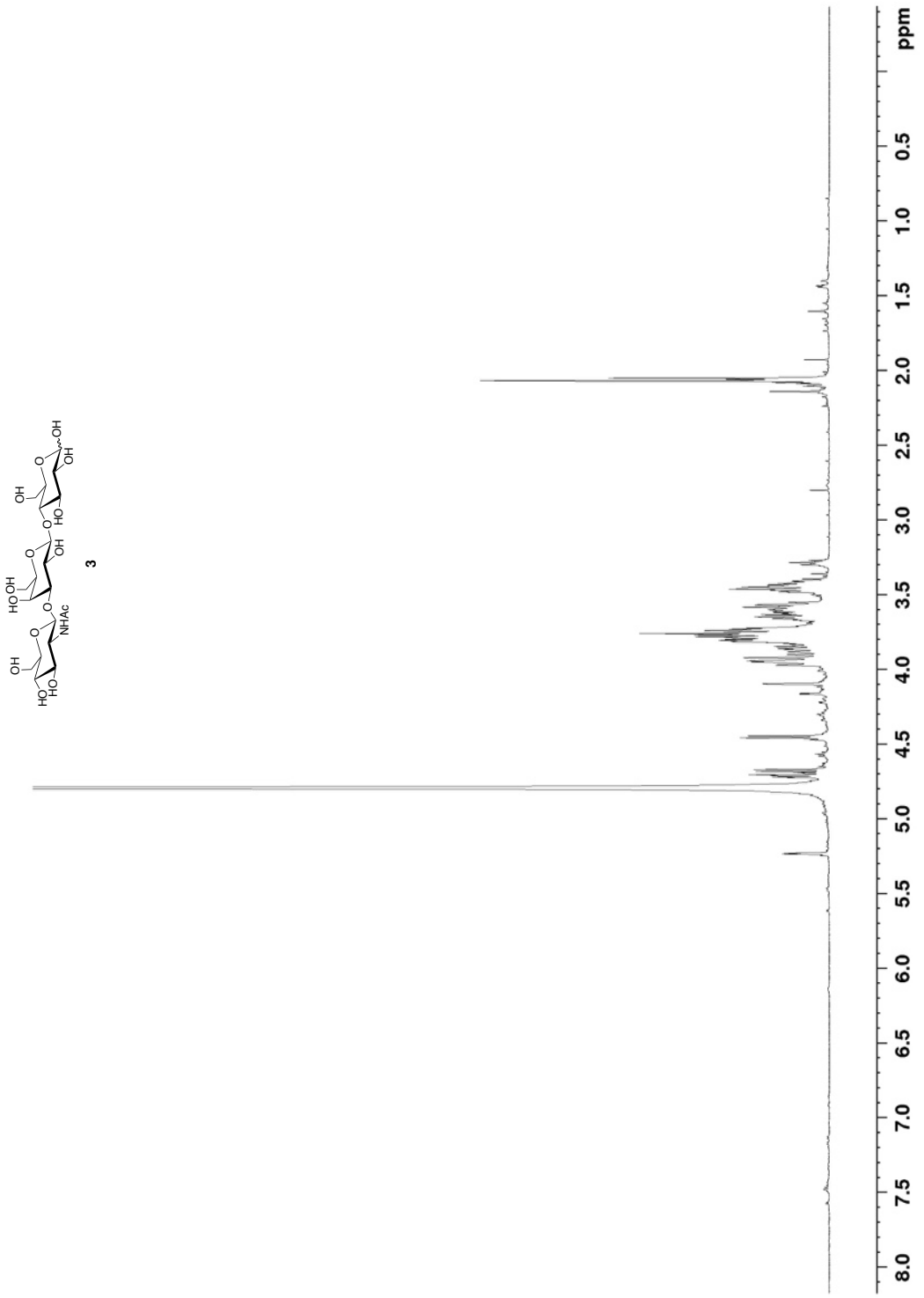


Figure A3. 77 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 3

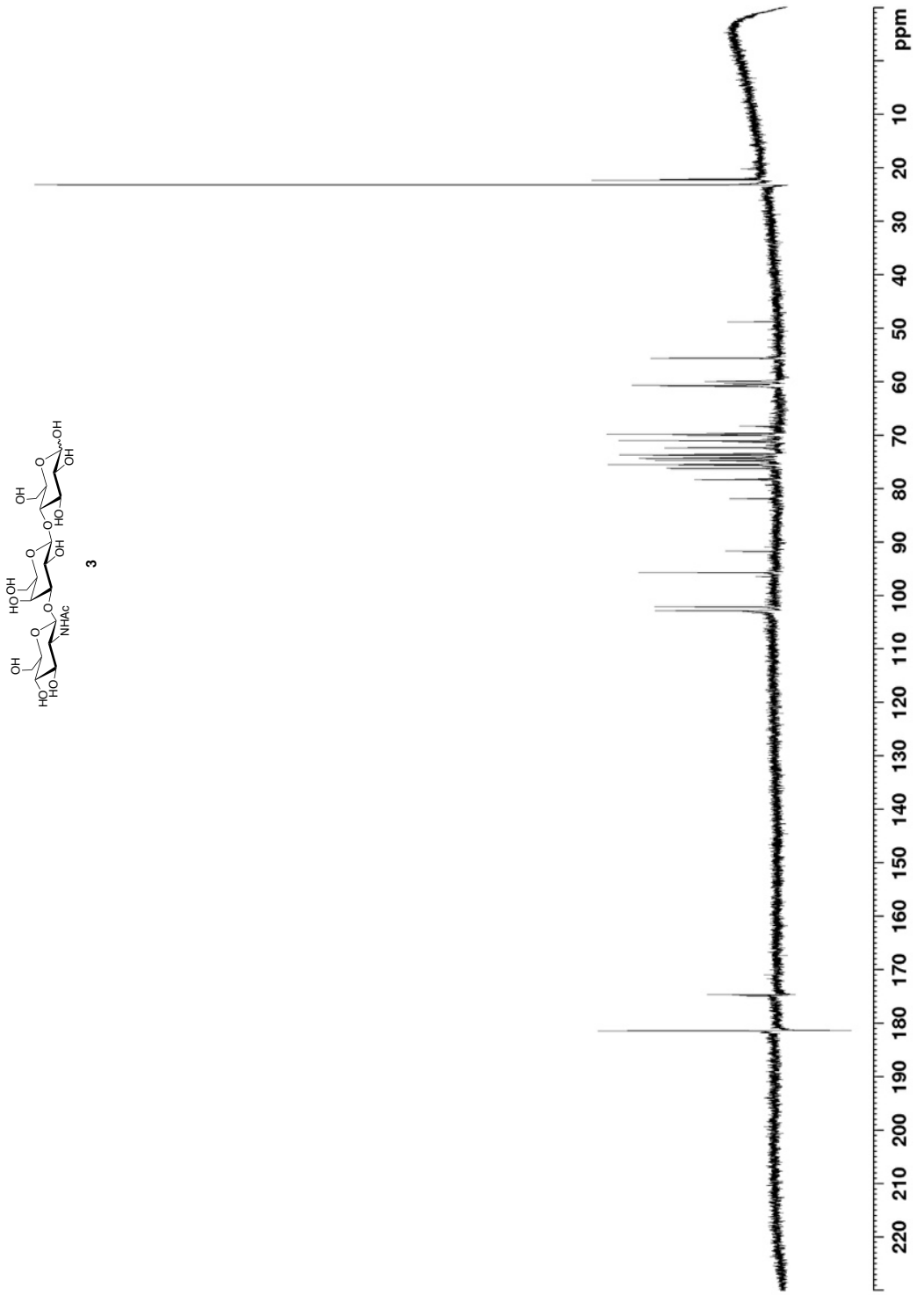


Figure A3. 78 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 3

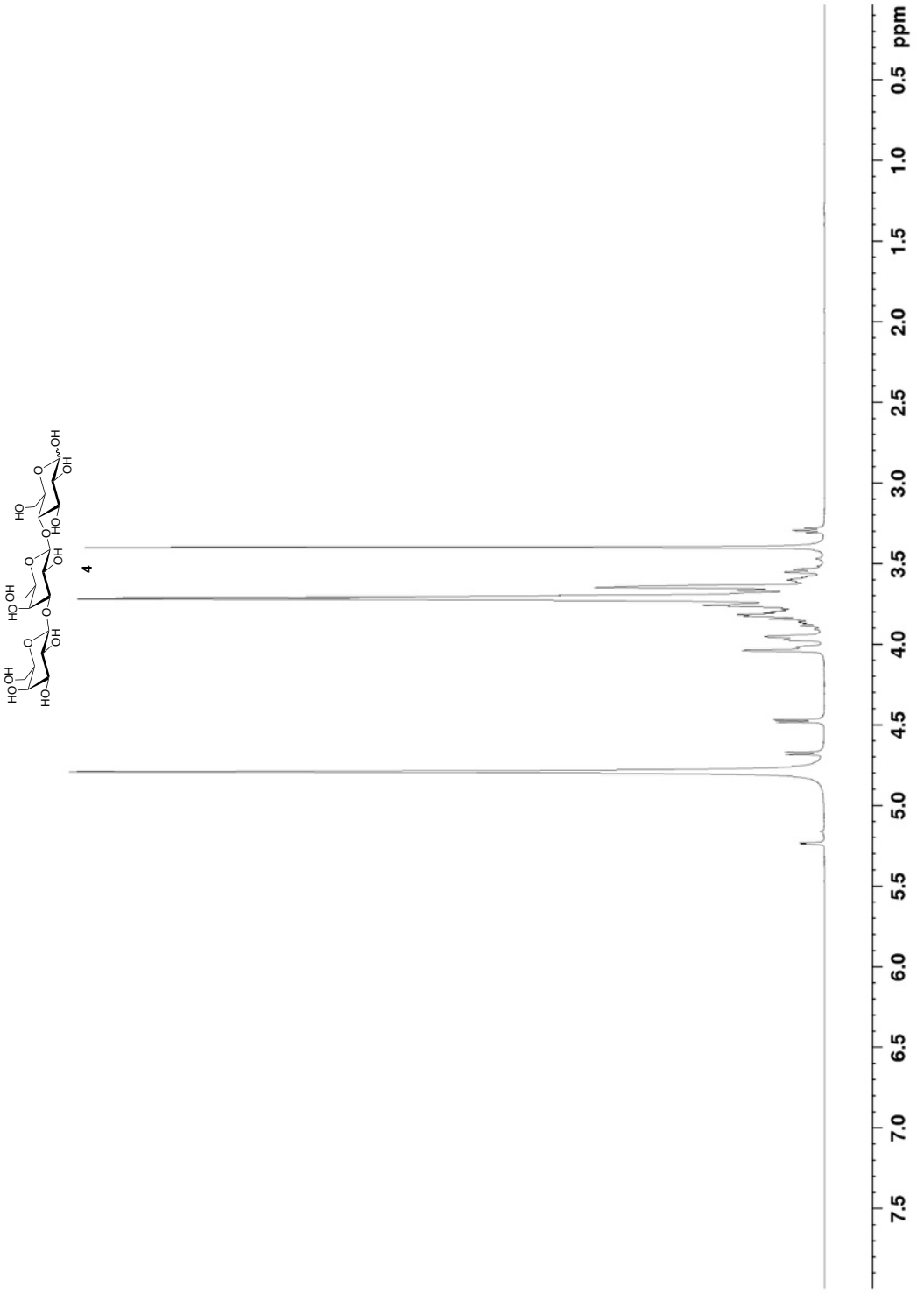


Figure A3. 79 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 4

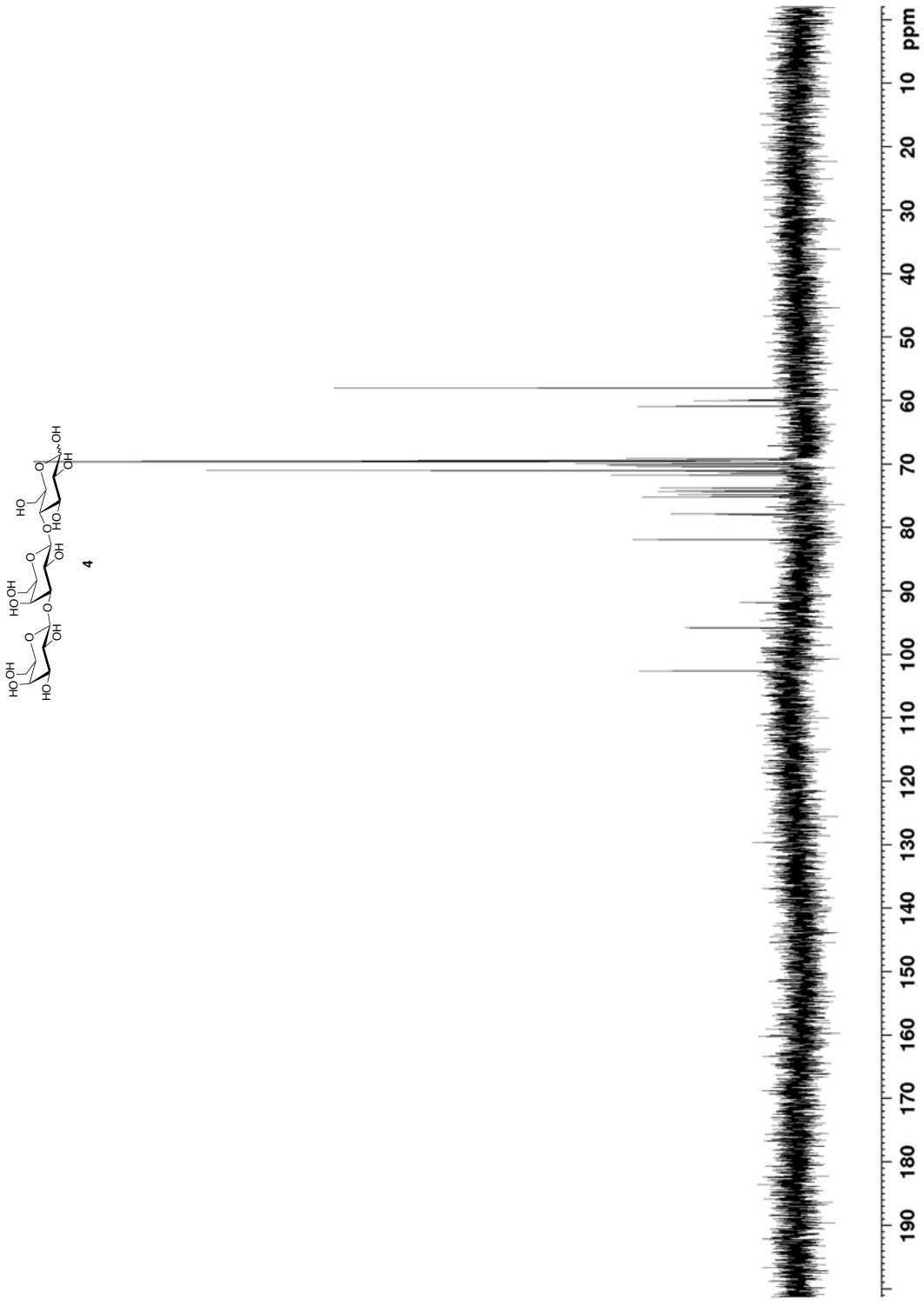


Figure A3. 80 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 4

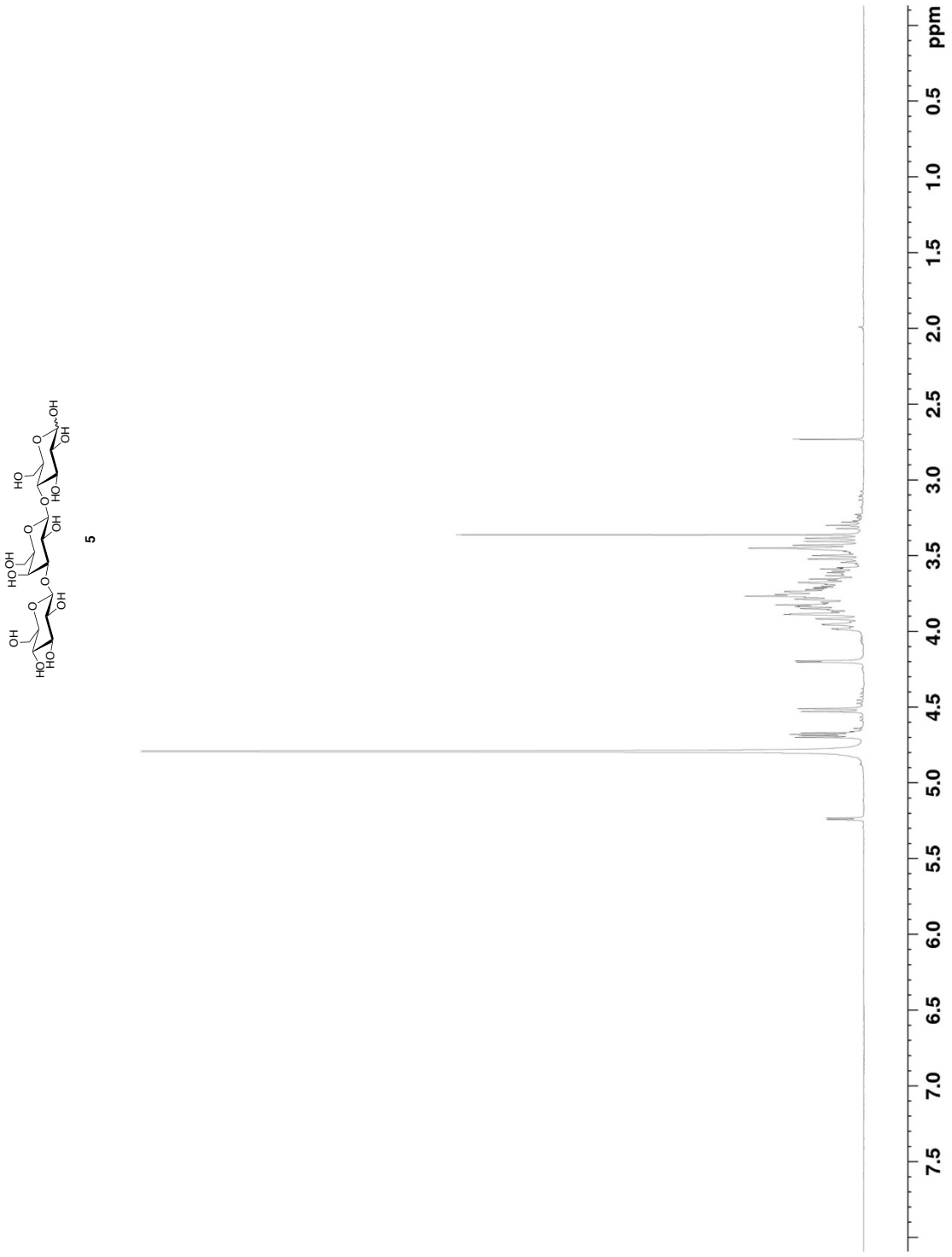


Figure A3. 81 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 5

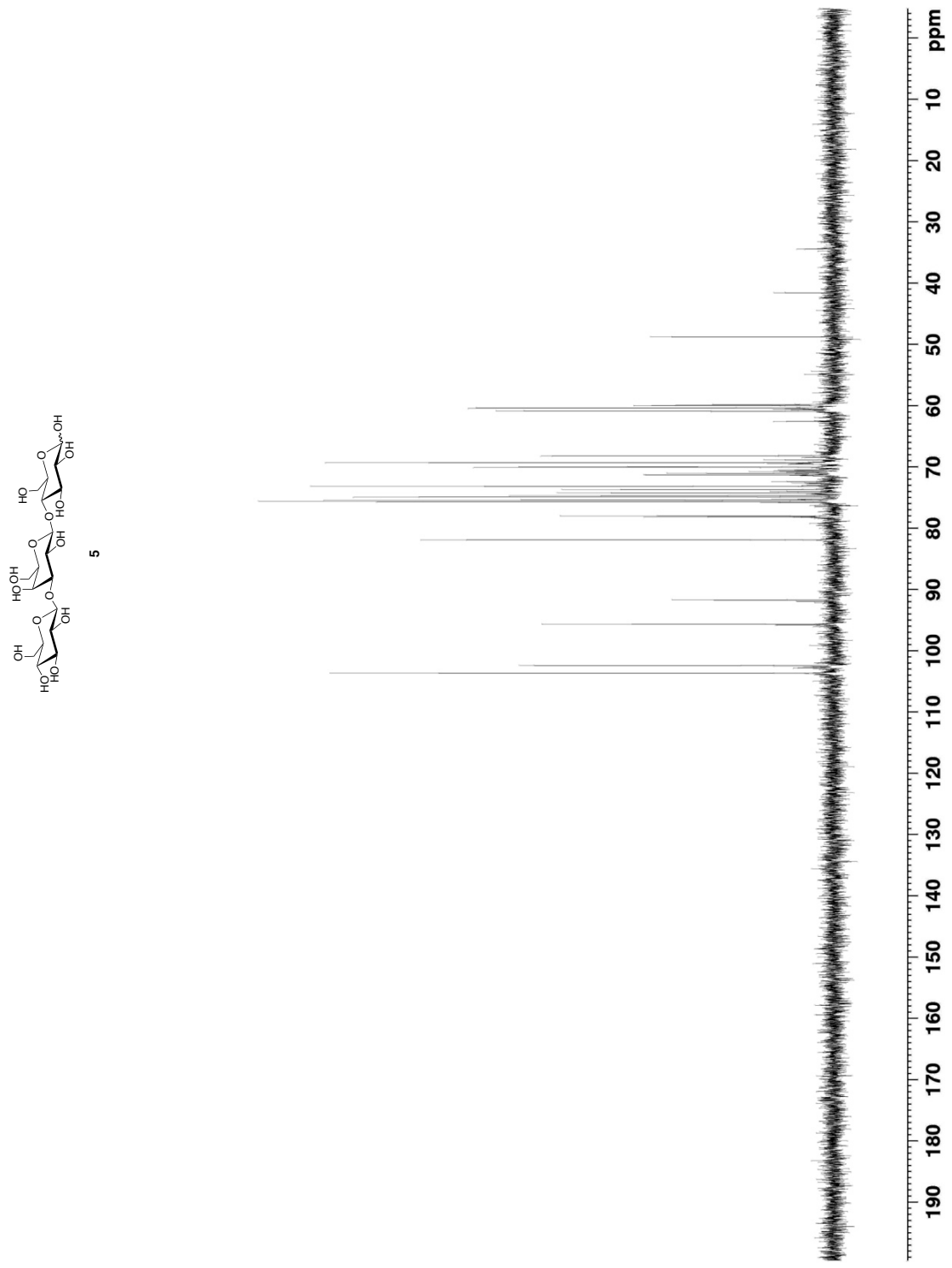


Figure A3. 82 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 5

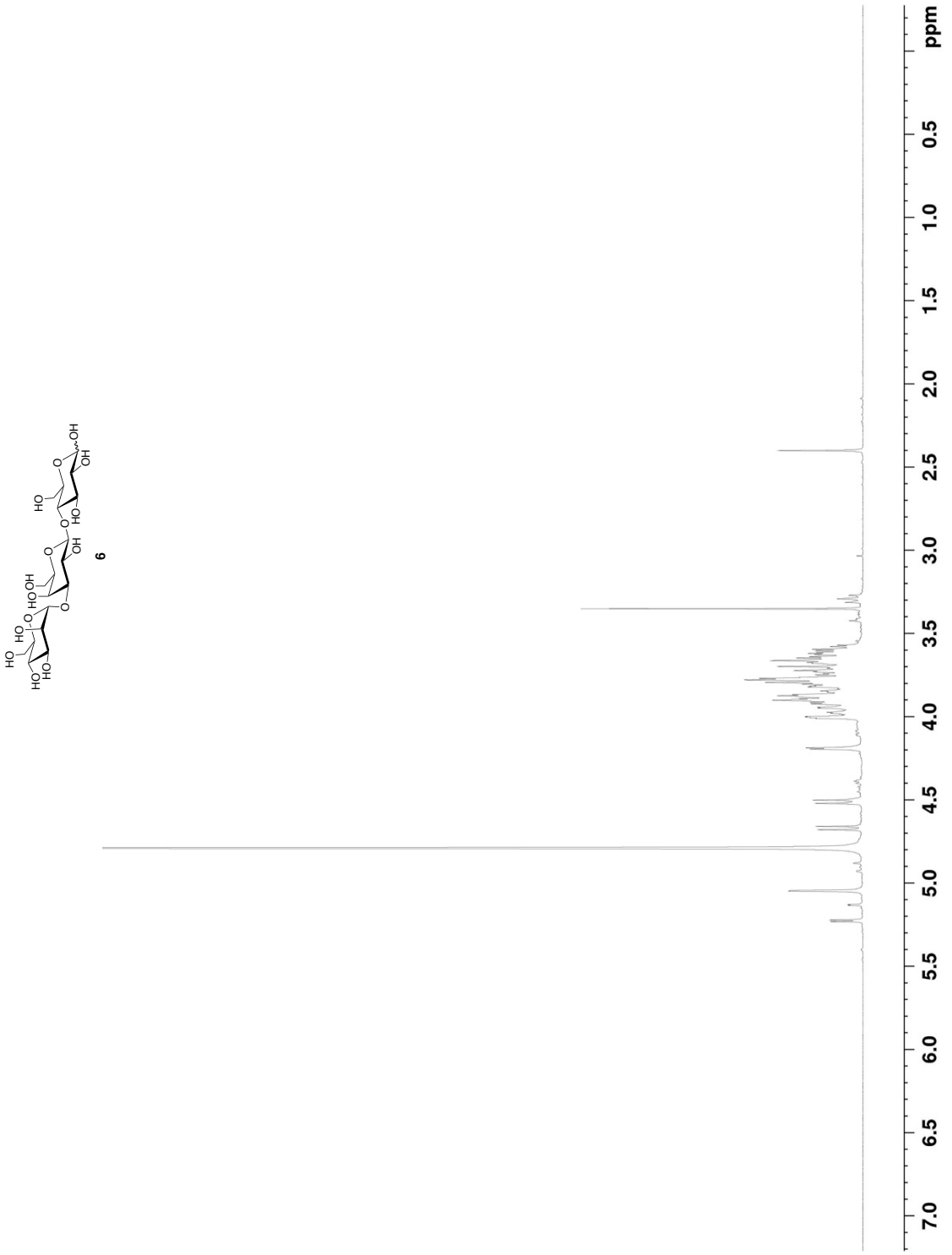


Figure A3. 83 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 6

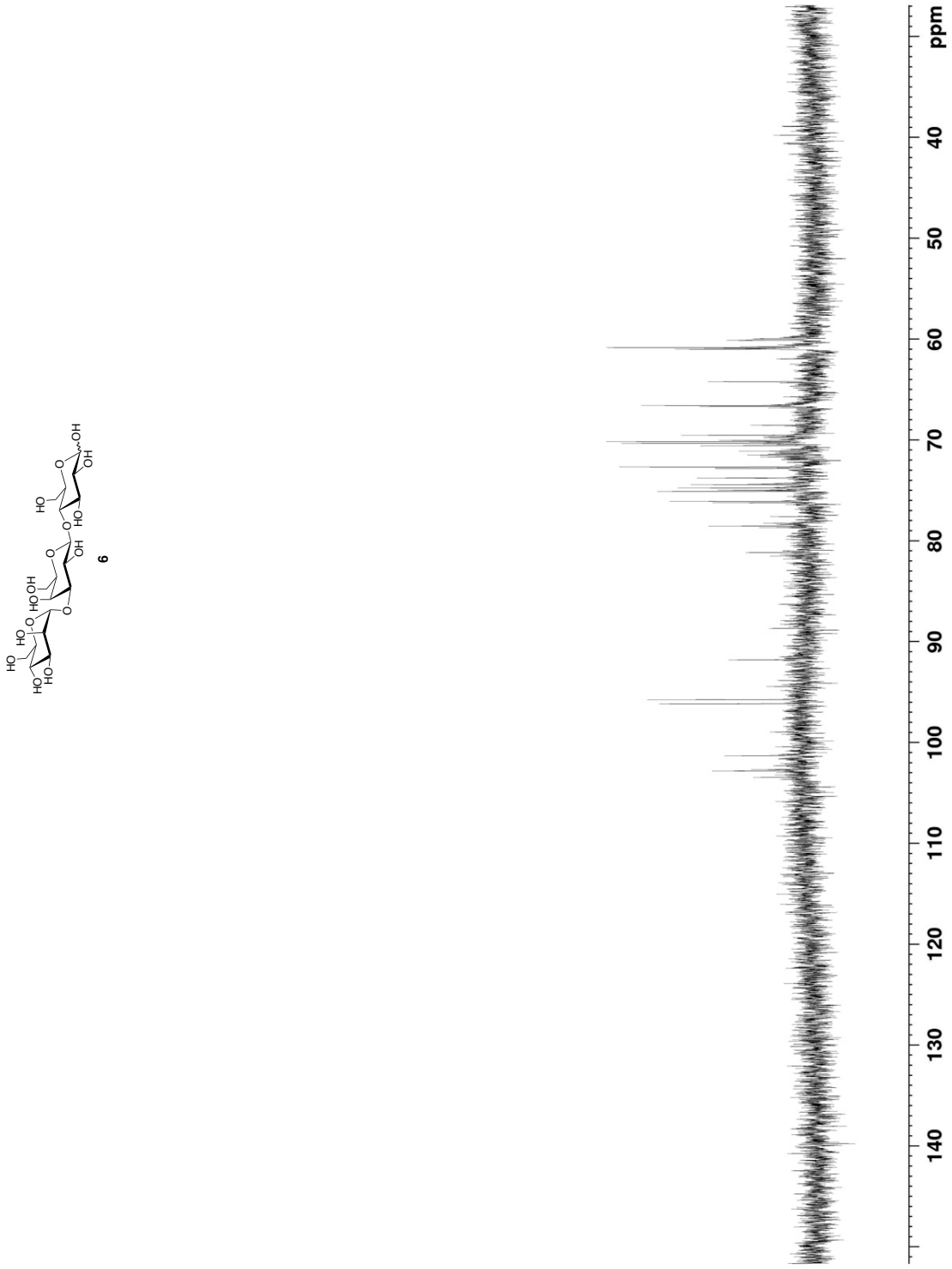


Figure A3. 84 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 6

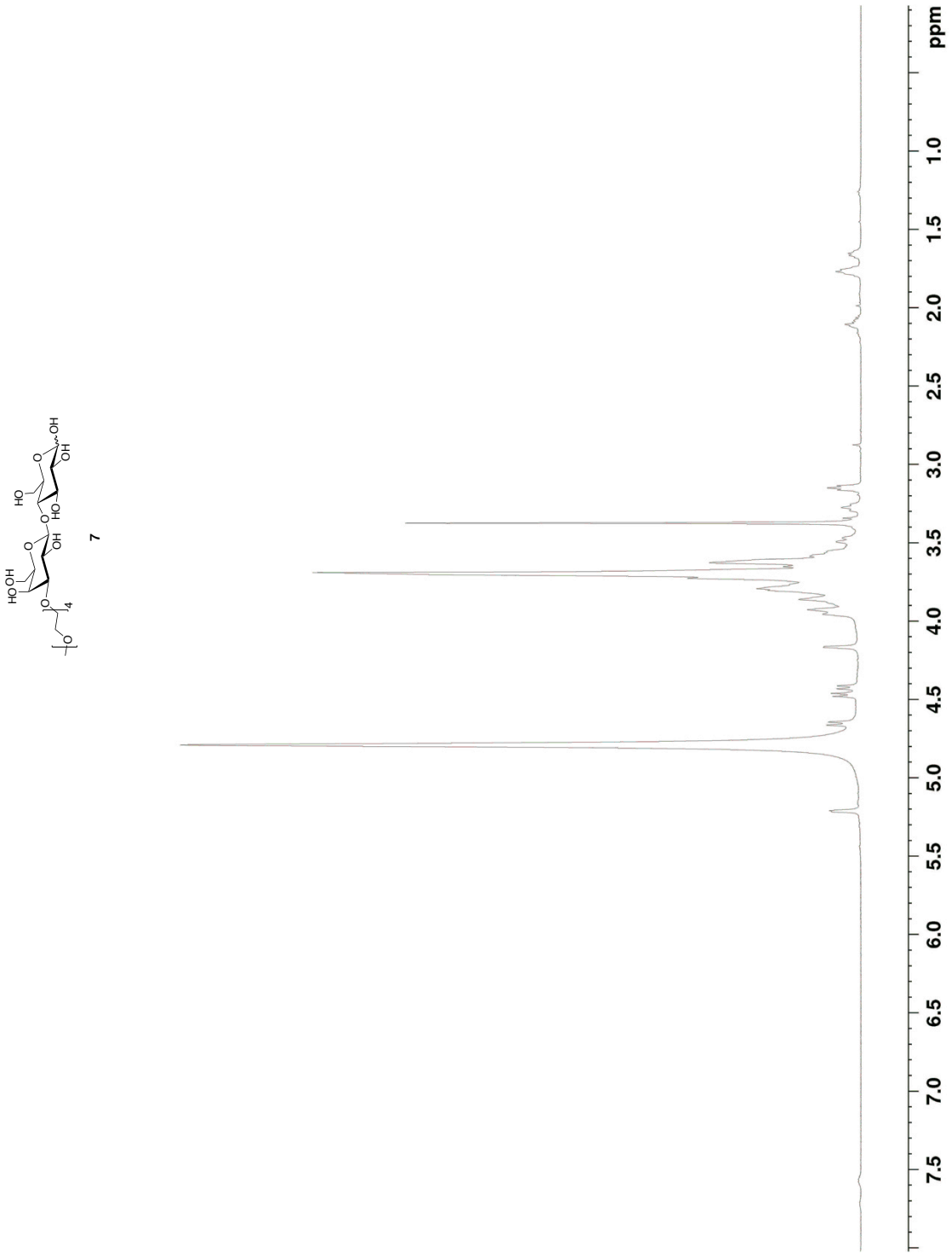


Figure A3. 85 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of compound 7

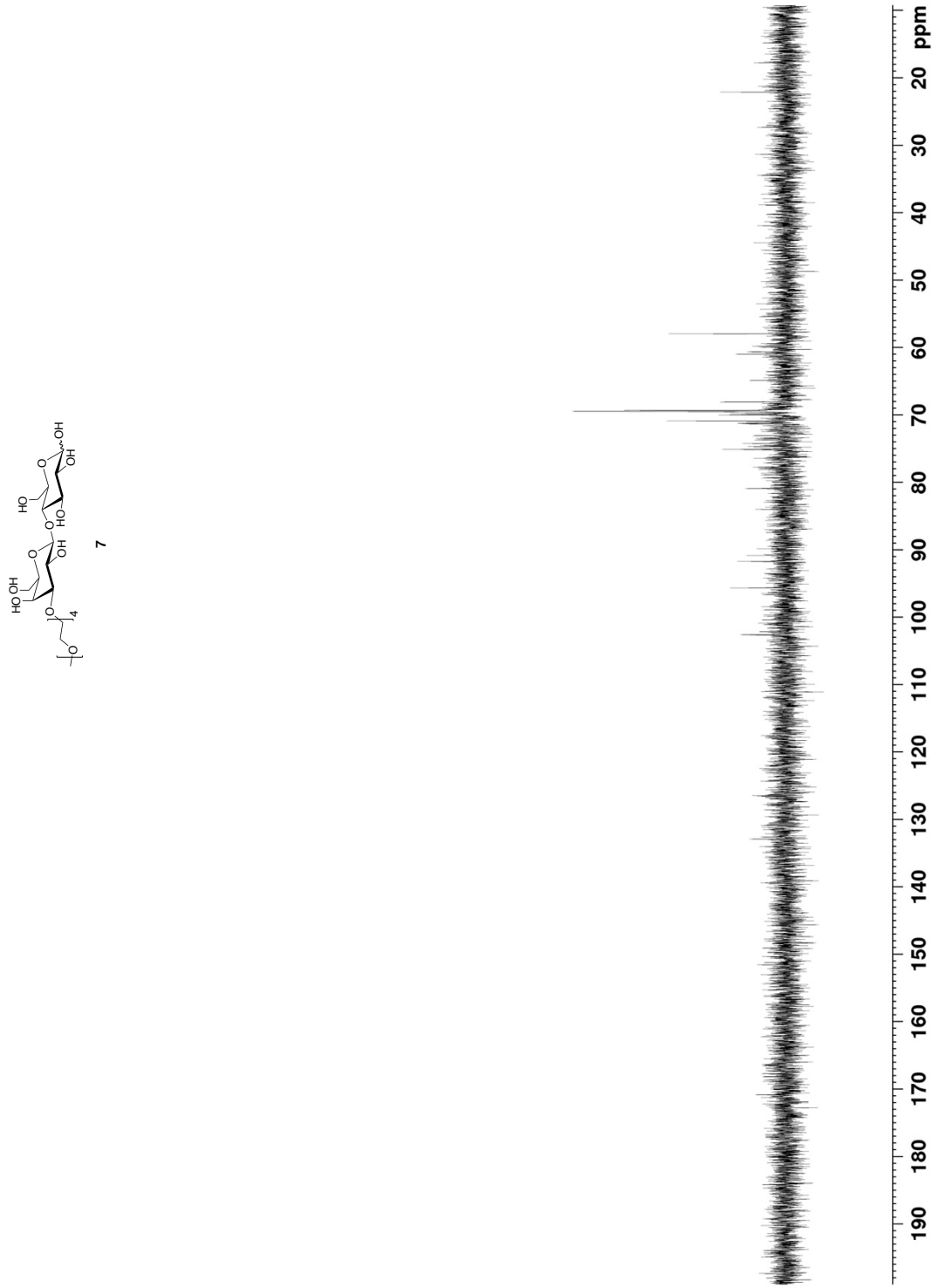


Figure A3. 86 ^{13}C NMR spectra (100 MHz, $CDCl_3$) of compound 7

CHAPTER V

Conclusions

In this dissertation, data revealing the antimicrobial and anti-biofilm activities of HMOs against GBS was presented. Furthermore, a potential relationship between fucosylated HMOs and antimicrobial activity was reported. These results call for additional studies to broaden the understanding of this activity.

First, these findings necessitate the elucidation of a mechanism of action for both the antimicrobial and anti-bioiflm activity of HMOs. While we are only able to hypothesize mechanisms at this stage, our hypotheses can serve as a starting point for future investigations. As mentioned previously, alterations in carbohydrate catabolism, enzyme inhibition that leads to gene regulation, or excess polysaccharide-induced biofilm modifications are plausible mechanisms. These hypotheses can be tested by monitoring the carbohydrate catabolism and metabolism through metabolomics and media characterization and genetic analysis. Analyzing the media for a difference in metabolites being released or any HMOs remaining after a period of growth will inform if the bacteria uses HMOs as a carbon source and if their catabolism or metabolism changes compared to a control. Another avenue that could be used to probe the mechanism is RNA sequencing and comparison of gene regulation between GBS grown in the presence or absence of HMOs. This will provide insight into the mechanisms that GBS employs to survive in the presence of HMOs. Musser and coworkers performed similar transcriptome sequencing studies for GBS (strain NEM316, serotype III) grown in media or human amniotic fluid.[1] While many genes were up or downregulated, their study revealed that genes for carbohydrate intake and metabolism are controlled by the growth media. We would expect to see changes in similar types of genes because

our study would include carbohydrates as part of one growth condition. For the genetic sequencing, it would be of particular interest to use the strain Bode and co-workers used in making their mutant library, as this could reveal if that particular gene (*gbs0738*/*gbs0408*) was up or down regulated when GBS is grown in the presence of HMOs.

As a continuation of the individual entity screens, it would be fitting to test other oligosaccharides present in fraction 7 of the fractionated HMOs from donor 42. We only explored trisaccharides that were present in the mixture along with related congeners. However, other longer-chain HMOs such as LNT, LNnT, and their fucosylated derivatives have masses corresponding to those observed in mass spectrometry characterization of the fraction. To support the viability of this approach, Bode and coworkers have tested several HMOs, including LNT, LNnT, LNnH, LNFPI, and LNFPV, and found that LNT and LNFP-I have antimicrobial activity. While previous studies have only measured the antimicrobial activity of HMOs, further studies should be done to look at the anti-biofilm activity of these compounds. The anti-biofilm activity seen by 3'-PEGylated lactose warrants further investigations into PEGylated derivatives of varying lengths and other ether polymers for their anti-biofilm activity. Additionally, because two negatively charges polymers, colominic acid and dextran sulfate, exhibited antimicrobial activity, designing a PEG derivative with sulfates or other charged moieties would be of interest. It is interesting to note that singly sialylated derivatives such as 3'-SL and 6'-SL had no effect on the growth but were able to inhibit biofilm formation. Perhaps the multiple negative charges are important for the antimicrobial activity seen by colominic acid and dextran sulfate.

We have tested whole HMO extracts, fractionated HMOs, and individual entities for their activity against GBS. As we saw a decrease in inhibitory activity for individual compounds

compared to pools of HMOs isolated from human milk, and we postulated this was due to the synergistic activity of HMOs, further work testing combination of individual entities could provide a framework for which compounds participate in synergistic activity.

Additionally, while HMOs act *in vitro* to affect the growth and biofilm production of GBS, it is important to study how HMOs act *in vivo* or in concert with elements of the innate immune system. HMOs have been shown to prevent the binding of other pathogens to host epithelial cells, such LNT in *E. histolytica* infections, LNnT and LSTc for *S. pneumoniae*, and fucosylated species for *C. jejuni*.[2-4] Similar studies to assay the ability of HMOs to prevent GBS binding to host epithelial cells would provide knowledge about the *in vivo* activity of HMOs to inhibit GBS pathogenesis.

A final area of inquiry is in antibiotic resistance. Over use and misuse of antibiotics has led to the development of antibiotic-resistant bacteria.[5] In fact, the CDC estimates that over 30% antibiotics prescribed are unnecessary, as many antibiotics are prescribed for viral infections.[6, 7] Furthermore, antibiotics are the most frequently prescribed medication to children.[8] While campaigns by the CDC and AAP have resulted in a decrease in pediatric antibiotic use, further improvement can be made in approaches for the use of broad- versus narrow-spectrum antibiotics.[9-14] While broad spectrum antibiotics are central to treating bacterial infections, their use promotes resistance evolution across species, and is a primary cause of microbiome dysbiosis.[15-17] In contrast, narrow-spectrum antibiotics are highly valuable and advantageous over broad-spectrum antibiotics due to their lower susceptibility to resistance development, decreased collateral damage to the host microbiome, and decreased development of antibiotic-associated colitis.[18] Given the growing threats to health posed by antibiotic resistance, the use of narrow spectrum antibiotics are

an urgent priority.[19,20] As reported by Bode and coworkers and our lab, it appears as though HMOs possess narrow-spectrum activity against GBS, though we found minimal antimicrobial activity was seen against *Acinetobacter baumannii*.[21] We have begun a program assessing the ability of HMOs to act synergistically with several common antibiotics to lower their MIC related to GBS growth. The first inquiry along these lines was measuring the effect of HMOs with polymyxin B, an antimicrobial peptide, and we observed that when HMOs from donor 43 were dosed with polymyxin B, complete growth inhibition of the colony occurred. Next, we dosed HMOs and antibiotics, and found that HMOs are able to significantly reduce the MIC of several antibiotics by 4-fold or higher. While most MIC-reduction activity was seen with penicillins and gentamycin, HMOs were able to reduce the MICs of both erythromycin and clindamycin in at least one strain. This is encouraging as GBS has begun to develop resistance to erythromycin and clindamycin. While it appears that HMOs may serve as a line of defense to rescue some antibiotics, it would also be beneficial to determine if GBS can develop resistance to HMOs themselves. It is unlikely that this resistance would develop as infants colonized with GBS have long received HMOs through breastfeeding and clinical isolates of GBS are still susceptible to the antimicrobial and anti-biofilm activity of HMOs, suggesting that if GBS were going to develop resistance it already would have.

To complement their antimicrobial and anti-biofilm properties, HMOs are themselves nontoxic at any concentration and are well-known to aid in proper neonate microbiome development. As such, the antimicrobial and anti-biofilm properties of HMOs position them to become tools to combat infectious diseases.

In summary, while we have established the antimicrobial and anti-biofilm activity of

HMOs against GBS, much work to detail the mechanisms by which this activity occurs remain. The outlook for developing the use of human milk oligosaccharides as a defense against Group B strep remains positive.

References

1. Sitkiewicz, I.; Green, N. M.; Guo, N.; Bongiovanni, A. M.; Witkin, S. S.; Musser, J. M., Transcriptome adaptation of group B Streptococcus to growth in human amniotic fluid. *PLoS One* **2009,** *4*, e6114.

2. Jantscher-Krenn, E.; Lauwaet, T.; Bliss, L. A.; Reed, S. L.; Gillin, F. D.; Bode, L., Human milk oligosaccharides reduce Entamoeba histolytica attachment and cytotoxicity in vitro. *Br J Nutr* **2012,** *108*, 1839-46.

3. Idanpaan-Heikkila, I.; Simon, P. M.; Zopf, D.; Vullo, T.; Cahill, P.; Sokol, K.; Tuomanen, E., Oligosaccharides interfere with the establishment and progression of experimental pneumococcal pneumonia. *J Infect Dis* **1997,** *176*, 704-12.

4. Crane, J. K.; Azar, S. S.; Stam, A.; Newburg, D. S., Oligosaccharides from human milk block binding and activity of the Escherichia coli heat-stable enterotoxin (STa) in T84 intestinal cells. *J Nutr* **1994,** *124*, 2358-64.

5. Facts about Antibiotic Resistance. https://www.cdc.gov/antibiotic-use/community/about/fast-facts.html (March 22, 2018),

6. Nyquist, A. C.; Gonzales, R.; Steiner, J. F.; Sande, M. A., Antibiotic prescribing for children with colds, upper respiratory tract infections, and bronchitis. *JAMA* **1998,** *279*, 875-7.

7. Fleming-Dutra, K. E.; Hersh, A. L.; Shapiro, D. J.; et al., Prevalence of inappropriate antibiotic prescriptions among us ambulatory care visits, 2010-2011. *JAMA* **2016,** *315*, 1864-1873.

8. Hersh, A. L.; Shapiro, D. J.; Pavia, A. T.; Shah, S. S., Antibiotic prescribing in ambulatory pediatrics in the United States. *Pediatrics* **2011,** *128*, 1053-61.

9. Sharland, M.; Pulcini, C.; Harbarth, S.; Zeng, M.; Gandra, S.; Mathur, S.; Magrini, N.; Use, s. W. E. C. S., Classifying antibiotics in the WHO Essential Medicines List for optimal use-be AWaRe. *Lancet Infect Dis* **2018,** *18*, 18-20.

10. Demirjian, A.; Sanchez, G. V.; Finkelstein, J. A.; Ling, S. M.; Srinivasan, A.; Pollack, L. A.; Hicks, L. A.; Iskander, J. K., CDC Grand Rounds: Getting Smart About Antibiotics. *Mmwr-Morbid Mortal W* **2015,** *64*, 871-873.

11. Goode, A. M.; Roberts, R. M., Pharmacists and physicians get smart about antibiotics: A prescription for change. *J Am Pharm Assoc* **2014,** *54*, 324-+.

12. In *Antibiotic Resistance: Implications for Global Health and Novel Intervention Strategies: Workshop Summary*: Washington (DC), 2010.

13. In *Antimicrobial Resistance: Issues and Options: Workshop Report*, Harrison, P. F.; Lederberg, J., Eds.: Washington (DC), 1998.

14. Finkelstein, J. A.; Stille, C.; Nordin, J.; Davis, R.; Raebel, M. A.; Roblin, D.; Go, A. S.; Smith, D.; Johnson, C. C.; Kleinman, K.; Chan, K. A.; Platt, R., Reduction in antibiotic use among US children, 1996-2000. *Pediatrics* **2003,** *112*, 620-627.

15. Costelloe, C.; Metcalfe, C.; Lovering, A.; Mant, D.; Hay, A. D., Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. *Brit Med J* **2010,** *340*.

16. Canning, D. A., Previous Antimicrobial Exposure is Associated With Drug-Resistant Urinary Tract Infections in Children Editorial Comment. *J Urology* **2011,** *185*, 682-682.

17. Paschke, A. A.; Zaoutis, T.; Conway, P. H.; Xie, D. W.; Keren, R., Previous Antimicrobial Exposure Is Associated With Drug-Resistant Urinary Tract Infections in Children. *Pediatrics* **2010,** *125*, 664-672.

18. Maxson, T.; Mitchell, D. A., Targeted Treatment for Bacterial Infections: Prospects for Pathogen-Specific Antibiotics Coupled with Rapid Diagnostics. *Tetrahedron* **2016,** *72*, 3609-3624.

19. Melander, R. J.; Zurawski, D. V.; Melander, C., Narrow-spectrum antibacterial agents. *Medchemcomm* **2018,** *9*, 12-21.

20. Melander, R. J.; Melander, C., The Challenge of Overcoming Antibiotic Resistance: An Adjuvant Approach? *ACS Infect Dis* **2017,** *3*, 559-563.

21. Lin, A. E.; Autran, C. A.; Szyszka, A.; Escajadillo, T.; Huang, M.; Godula, K.; Prudden, A. R.; Boons, G. J.; Lewis, A. L.; Doran, K. S.; Nizet, V.; Bode, L., Human milk oligosaccharides inhibit growth of group B Streptococcus. *J Biol Chem* **2017**.