Reconstitution of the Final Step of Peptidoglycan Assembly in Staphylococcus aureus

Reconstitution of the Final Step of Peptidoglycan Assembly in *Staphylococcus aureus*

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Abstract

Bacterial peptidoglycan (PG) is an exoskeleton structure that maintains cell shape and protects cells from lysis. Peptidoglycan is essential in bacteria but is not found in mammalian cells. Therefore, it is the target for several classes of antibiotics, including the beta-lactam family. Beta-lactams target the transpeptidase (TP) domain of penicillin-binding proteins (PBPs), the enzymes responsible for the final step of peptidoglycan biosynthesis. Mutations of transpeptidases in *Staphylococcus aureus* (*S. aureus*) have been implicated in beta-lactam resistance in the clinic (commonly known as MRSA infections). Despite the fact that PBPs are important antibiotic targets, there have been no direct assays to monitor their enzymatic activity, primarily due to inaccessibility to appropriate substrates. The PG precursor, Lipid II, is required to study transpeptidase activity. Lipid II contains a glycopeptide attached to a pyrophosphate lipid containing 55 carbons. It has poor physical properties and is present in low abundance in bacteria.

This thesis describes the reconstitution of peptidoglycan assembly in *Staphylococcus aureus* by the essential Class A PBP, PBP2. This was enabled by several key advances, which are also described. The first advance is the discovery that PBP4, a low-molecular weight PBP in *S. aureus*, can use a Lipid II analogue as a transpeptidation substrate. It can incorporate biotin-D-Lys (BDL) and other non-canonical D-amino acids into the terminal position of the stem peptide in Lipid II. BDL labeling of Lipid II with *S. aureus* PBP4 has enabled the second advance: the direct detection of native Lipid II extracted from bacteria. This has facilitated elucidation of

cellular mechanisms of antibiotics that target cell wall biosynthesis. The third advance is a general strategy to accumulate and isolate native Lipid II in bacteria in useful quantities. Access to substantial quantities of native *S. aureus* Lipid II has enabled reconstitution of PBP2 transpeptidase activity, as well as characterization of several beta-lactam antibiotics by monitoring enzymatic inhibition.

In sum, this work establishes important tools for studying enzymatic mechanisms of bacterial transpeptidases and for characterizing inhibitors that target bacterial peptidoglycan biosynthesis.

Table of Contents

Abstract iii

Table of contents v

List of Figures viii

List of Table xii

List of Schemes xii

Glossary of Abbreviations xiii

Acknowledgements xv

Chapter One: Introduction to peptidoglycan biosynthesis in *Staphylococcus aureus*

1.1 Penicillin, the wonder drug 2

1.2 The bacterial cell wall pathway 3
- 1.2.1 Formation of the Park nucleotide 5
- 1.2.2 Formation of the lipid-linked intermediates 7
- 1.2.3 Peptidoglycan (PG) assembly 13

1.3 The cellular roles of *S. aureus* penicillin-binding proteins (PBPs) 17

1.4 Resistance in methicillin-resistant *S. aureus* (MRSA) 22
- 1.4.1 An acquired resistant PBP, PBP2a in MRSA 22
- 1.4.2 Factors influencing methicillin resistance 25
- 1.4.3 Chemical genetic approaches to combat MRSA 27

1.5 Lipid II is required for enzymatic studies of PBPs 27
- 1.5.1 Current approaches of Lipid II preparation 28
- 1.5.2 Mechanistic insights into peptidoglycan glycosyltransferase (PGT) activities 32
- 1.5.3 Existing knowledge on PBP transpeptidase (TP) activities 35

1.6 Perspectives 36

1.7 References 38

Chapter Two: Exploiting the transpeptidase activity of PBP4 to detect peptidoglycan precursors in cells and to elucidate the mechanism of antibiotics that target cell wall biosynthesis

2.1 Introduction 59
- 2.1.1 *S. aureus* PBP4, a peculiar PBP 59
- 2.1.2 Challenges with studying the cellular pools of Lipid II in bacteria 62

2.2 Purification and characterization of *S. aureus* PBP4 64

2.3 Assessment of *S. aureus* PBP4 *in vitro* activity 65

2.4 Assessment of *S. aureus* PBP4 *in vivo* activity 67

2.5 Analysis of *S. aureus* PBP4 substrate specificity *in vitro* 69

2.6 Detection of the cellular levels of Lipid II in bacteria 73

2.7 Elucidation of the cellular mechanism of action for lysobactin 75

2.8 Conclusion 77

2.9 Materials and Methods
2.9.1 Materials 79
2.9.2 General methods 80
2.9.3 Plasmid construction 80
2.9.4 Overexpression and purification of *S. aureus* PBP4 and mutant 81
2.9.5 Bocillin-FL binding assay 82
2.9.6 LC/MS protocol for muropeptide analysis 82
2.9.7 Microscopy imaging analysis 84
2.9.8 Preparation of BDL probe 84
2.9.9 Incorporation of functionalized D-amino acids into Lipid I and Lipid II 84
2.9.10 Western blot detection of BDL-Lipid II *in vitro* 86
2.9.11 Extraction of cellular lipid-linked PG precursors and Western blot detection 86

2.10 References 88

Chapter Three: Preparation of native *Staphylococcus aureus* Lipid II

3.1 Introduction 97

3.2 Chemical synthesis of *S. aureus* Lipid II
3.2.1 Synthesis of stem pentapeptide containing variable glycine branches 99
3.2.2 Coupling reactions to make Lipid I and Lipid II variants 104

3.3 A facile method to obtain native *S. aureus* Lipid II 106
3.3.1 Estimation of *S. aureus* Lipid II quantity 111

3.4 A general approach to obtain bacterial Lipid II 112

3.5 Conclusion 114

3.6 Materials and Methods
3.6.1 Experimental details of synthesis 115
3.6.2 Small-scale extraction of *S. aureus* Lipid II 130
3.6.3 Large-scale extraction of *S. aureus* Lipid II 131
3.6.4 Thin layer chromatography analysis of phospholipids 132
3.6.5 Western blot analysis of BDL-labeled Lipid II 133
3.6.6 LC/MS analysis of delipidated *S. aureus* Lipid II 133
3.6.7 Quantifications of delipidated *S. aureus* Lipid II 134

3.7 References 135

Chapter Four: Reconstitution of peptidoglycan assembly by *Staphylococcus aureus* PBP2

4.1 Introduction 139
4.1.1 PBP2 is the essential Class A PBP in *S. aureus* 139
4.1.2 Traditional competition binding assays to study beta-lactams 142

4.2 Purification of *S. aureus* PBP2 143

4.3 Reconstitution of the PGT and TP activities of *S. aureus* PBP2 145

4.4 Assessing the inhibitory effects of beta-lactams 148

4.5 Investigating the substrate specificity of *S. aureus* PBP2 151

4.6 Conclusion and future studies 152

4.7 Materials and methods
4.7.1 General methods 154
4.7.2 Cloning of *S. aureus* PBP2[M59-S716] and mutants 154
4.7.3 Overexpression and purification of *S. aureus* PBP2 and mutants 155
4.7.4 LC/MS assay analysis of *S. aureus* PBP2 activity *in vitro* 156
4.7.5 Degradation of isolated *S. aureus* sacculi 157
4.7.6 Western blot analysis of *S. aureus* PBP2 TP activity 158
4.7.7 Studies on beta-lactam inhibition of PBP2 TP activity 159

4.8 References 160

Appendix One: Mass spectrometry (MS) analysis for studies in Chapter Two 164

Appendix Two: NMR and MS data for compounds in Chapter Three 170

Appendix Three: MS data and Western blot analysis for studies in Chapter Four 192

List of Figures

Figure 1.1. General structure of the penicillin family of antibiotics. 2

Figure 1.2. Structure of the Park nucleotide in *S. aureus.* 6

Figure 1.3. Structure of Lipid I and Lipid II intermediates identified in the cell-free system using the particulate enzymes from *S. aureus.* 8

Figure 1.4. The complete Lipid II structure in *S. aureus.* 11

Figure 1.5. Schematic showing pepetidoglycan (PG) biosynthesis in *S. aureus.* 15

Figure 1.6. The proposed mechanisms of transpeptidation (and hydrolysis) by PBPs and penicillin inhibition. 16

Figure 1.7. Beta-lactams show selective binding to *S. aureus* PBPs. 18

Figure 1.8. An allosteric regulation of *S. aureus* PBP2a activity is proposed. 25

Figure 1.9. The synthetic route to make Lipid I by Tsukamoto and Kahne. 30

Figure 2.1. Schematic of carboxypeptidase and transpeptidase activities in *S. aureus* PG assembly and Lipid II substrates. 60

Figure 2.2. Protein sequence alignments of representative LMW PBPs. 61

Figure 2.3. *S. aureus* PBP4 protein is purified to homogenity. 65

Figure 2.4. *S. aureus* PBP4 demonstrates TP activity *in vitro*. 67

Figure 2.5. *S. aureus* PBP4 has TP activity *in vivo*. 68

Figure 2.6. *S. aureus* PBP4 exhibits promiscuous TP activity *in vitro*. 70

Figure 2.7. LC/MS analysis of *S. aureus* PBP4-mediated D-amino acid exchange in Lipid II. 72

Figure 2.8. Schematic showing the versatility of PBP4 TP activity. 73

Figure 2.9. *S. aureus* PBP4 enables a simple assay to detect cellular Lipid II in bacteria. 75

Figure 2.10. Lysobactin treatment accumulates Lipid II in *S. aureus*. 77

Figure 3.1. Moenomcyin treatment accumulates Lipid II by 10-fold in *S. aureus.* 108

Figure 3.2. Preparative quantities of the native *S. aureus* Lipid II can be obtained via a two-step accumulation and extraction procedure. 109

Figure 3.3. Structural confirmation of isolated *S. aureus* Lipid II. 111

Figure 3.4. Structures of deplidated Lipid II species. 112

Figure 3.5. Structures of Lipid II species isolated from different bacteria. 114

Figure 4.1. Schematic depiction of *S. aureus* PG biosynthetic pathway. 140

Figure 4.2. Purification of *S. aureus* PBP2[M59-S716] construct and mutants. 144

Figure 4.3. *S. aureus* PBP2 uses native Lipid II to make cross-linked PG *in vitro*. 146

Figure 4.4. Beta-lactams inhibit *S. aureus* PBP2 TP activity. 150

Figure 4.5. *S. aureus* PBP2 demonstrates substrate specificity in transpeptidation. 152

Figure App1.1. *S. aureus* PBP4 shows TP activitiy *in vitro*. 165

Figure App1.2. *S. aureus* PBP4 shows TP activitiy *in vitro*. 166

Figure App1.3. Analysis of *S. aureus* PBP4 activities at varying concentrations of D-Tyr nucelophile. 167

Figure App1.4. *S. aureus* PBP4 efficiently incorproate D-amino acids into *m*-Dap-Lipid II and Lys-Lipid II *in vitro*. 168

Figure App1.5. *S. aureus* PBP4 incorporates functionalized D-amino acids into Lys-Lipid I and Lys-Lipid II *in vitro*. 169

Figure App2.1. ^{1}H NMR of Boc-L-Ala-D-*iso*Gln-OH. 171

Figure App2.2. ^{1}H NMR and LRMS of Boc-L-Ala-D-*iso*Gln-L-Lys(Cbz)-D-Ala-D-Ala-OMe (**1**). 172

Figure App2.3. ^{1}H NMR and LRMS of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-TFA)-D-Ala-

D-Ala-OMe (**3**). 173

Figure App2.4. ^{1}H NMR and LRMS of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (**4**). 174

Figure App2.5. ^{1}H NMR and LRMS of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**5**). 175

Figure App2.6. ^{1}H NMR and LRMS of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (**6**). 176

Figure App2.7. ^{1}H NMR and LRMS of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**7**). 177

Figure App2.8. ^{1}H NMR and LRMS of Cbz-D-*iso*Gln(Trt)-OH. 178

Figure App2.9. ^{1}H NMR of Boc-L-Ala-D-*iso*Gln(Trt)-L-Lys(-Cbz)-D-Ala-D-Ala-OMe (**9**). 179

Figure App2.10. ^{1}H NMR and LMRS of Boc-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (**10**). 180

Figure App2.11. ^{1}H NMR and LMRS of Boc-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**11**). 181

Figure App2.12. LRMS of protected Gly-Lipid I. 182

Figure App2.13. LRMS of Gly-Lipid I (**12**). 182

Figure App2.14. HRMS of Gly-Lipid I (**12**). 183

Figure App2.15. LRMS of protected Gly_3-Lipid I. 184

Figure App2.16. LRMS of Gly_3-Lipid I (**13**). 184

Figure App2.17. HRMS of Gly_3-Lipid I (**13**). 185

Figure App2.18. LRMS of protected Gly_5-Lipid I (**14**). 186

Figure App2.19. HRMS of Gly-Lipid II (**15**). 187

Figure App2.20. HRMS of Gly_3-Lipid II (**16**). 188

Figure App2.21. LC/MS/MS of delipidated *S. aureus* Lipid II. 189

Figure App2.22. Estimation of the amount of *S. aureus* Lipid II isolated from cells. 190

Figure App2.23. LC/MS analysis of delipidated Lipid II from different bacteria. 191

Figure App3.1. The TP domain of *S. aureus* PBP2 is responsible for PG cross-linking. 193

Figure App3.2. Beta-lactams inhibit *S. aureus* PBP2 TP activity *in vitro*. 194

List of Table

Table 1.1. Classification of PBPs in *S. aureus*. 21

List of Schemes

Scheme 3.1. Synthetic route of Gly_1-pentapeptide (**3**), Gly_3-pentapeptide (**5**), Gly_5-pentapeptide (**7**). 102

Scheme 3.2. Alternative routes attempted to avoid complication by the carboxamide in the stem pentapeptide. 104

Scheme 3.3. The general reaction sequence to afford Lipid I and Lipid II variants. 106

Glossary of Abbreviations

6-APA	6-aminopeicillanic acid
Ala	alanine
Bac	bacitracin
BDL	biotinylated-D-lysine
Bo-FL	Bocillin-FL
C35	heptaprenyl
C55	undecaprenyl
ACN	acetonitrile
CDI	1,1-carbonyldiimidazole
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CP	carboxypeptidase
DMSO	dimethyl sulfoxide
DMTMM	dimethoxytriazine-N-methylmorpholinium chloride
ECA	enterobacterial common antigen
ER	endoplasmic reticulum
FDL	fluorescent-D-lysine
Fos	fosfomycin
Gal	galactose
GlcNAc	N-acetyl-glucosamine
Gln	glutamine
Glu	glutamic acid

Gly	glycine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HMW	high-molecular weight
HPLC	high pressure liquid chromatography
Kan	kanamycin
LC/MS	liquid chromatography mass spectrometry
LDAO	N,N-dimethyldodecylamine N-oxide
LMW	low-molecular weight
LPII	Lipid II
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Lys	lysine
Lyso	lysobactin
m-Dap	*meso*-diaminopimelic acid
MmA	moenomycin
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MurNAc	N-acetyl-muramic acid
NMR	nuclear magnetic resonance
PBP	penicillin-binding protein
penG	penicillin G
PEP	phosphoenolpyruvate

PG	peptidoglycan
PGT	peptidoglycan glycosyltransferase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tar	targocil
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TM	transmembrane
TP	transpeptidase
Tris	tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate
UnP	undecaprenyl phosphate
Vanco	vancomycin
wt	wildtype
WTA	wall teichoic acid

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Chapter One: Introduction to peptidoglycan biosynthesis in *Staphylococcus aureus*

1.1 Penicillin, the wonder drug

The story of penicillin discovery is not an unfamiliar one. In 1928, Alexander Fleming made a serendipitous observation that a *penicllium* mold contamination on a petri dish containing *Staphylococcus aureus* (*S. aureus*) inhibited the growth of the bacteria.[1,2] The term, penicillin, was thus coined for the antibacterial substance produced by the *penicillium* mold.[3] Subsequent work by Howard Florey, Ernst Chain[a] and Norman Heatley contributed to the extraction and purification of penicillin from mold culture for systematic application.[4] Despite a lack of knowledge on its structure and mechanism of action at the time, penicillin's phenomenal antibacterial activity had been realized and harnessed by the 1940s, leading to its medicinal use as an important class of antibiotics in man.[5,6] The expeditious development of penicillin deemed extremely beneficial in those days when bacterial infection was the leading cause of deaths in the general population. Juxtaposed with the World War II, penicillin, produced in large quantities by several pharmaceutical companies in the United States, made an indelible mark in human history by saving lives of numerous people that were afflicted with wound infections in the battlefield.[1,7] The enormous demand of the antibiotic culminated during the war and led to a staggering annual production of penicillin of over 600 billion units per month.[8b] Penicillin has revolutionized modern medicine.

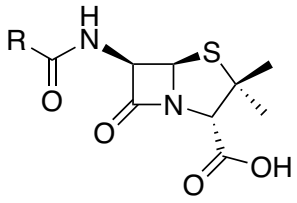

Figure 1.1. General structure of the penicillin family of antibiotics.

[a] Together with Fleming, Florey and Chain shared the Nobel Prize in Medicine in 1945 for the discovery of penicillin and its curative effect in various infectious diseases.
[b] One unit is equivalent to 0.6 μg of penicillin.

Shortly after the widespread use of penicillin in the general public, the chemical structure of penicillin was determined in 1945,[9,10] which in turn greatly facilitated the chemical syntheses of penicillin and its derivatives for activity optimization (Figure 1.1).[11] Semi-synthetic approaches making use of the natural precursor, 6-aminopenicillanic acid (6-APA), were also widely used to prepare diverse beta-lactam structures.[12-14] Penicillin is the founding member of the beta-lactam family, which represents the oldest yet the most successful class of antibiotics in history. Currently, beta-lactam drugs comprise more than half of the commercially available antibiotics in the market.[15]

1.2 The bacterial cell wall pathway

In addition to its glory as a miracle drug, penicillin has been a successful chemical probe in illuminating our understanding of bacterial physiology. In the extended efforts to understand the mechanism of penicillin, the cell wall biosynthesis--- a major metabolic pathway in bacteria was elucidated. In 1940, Gardner A. D. first noticed the striking morphological changes in a variety of bacteria when grown in dilute concentrations of penicillin.[16] Rod-shaped bacteria such as *Escherichia coli* (*E. coli*) became filamentous and grotesquely swollen, while *S. aureus* cells became spherically enlarged. [17,18] Such swelling and bulging in bacteria as a result of penicillin treatment led Duguid J. P. (1946) to predict that penicillin disrupts the formation of a cell surface structure that supports the cell,[19] even before the identification of the bacterial cell wall. In addition, the site of action of penicillin in bacteria was proposed to be on the cytoplasmic membrane, since exposing bacteria to radioactive penicillin resulted in strongly radiolabeled membranes.[20-22]

With the development of electron microscopic techniques in the 1940s, bacterial cell wall was shown as an encasing structure outside of the cytoplasmic membrane in bacteria.[23-25] Based on the observations that the isolated cell wall maintained bacterial shape, and that bacteria became spherical protoplasts after lysozyme[c] treatment in hypertonic sucrose solution, it was proposed that the bacterial cell wall is a rigid structure protecting the cell from rapturing under the high internal osmotic pressure.[26-29] In Gram-positive bacteria such as *S. aureus*, the cell wall is considerably thicker (~20 nm),[30] while Gram-negative bacteria have a much thinner cell wall (5 nm) in the periplasmic space.[31] Analysis of the cell wall composition revealed unusual features such as D-amino acids and *N*-acetyl-muramic acids (MurNAc), which were not observed in other metabolic processes in bacteria.[32] In general, the cell wall, also referred to as peptidoglycan (PG), is composed of glycan strands made of alternating *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) units, with a stem peptide attached on each MurNAc residue.[33] The identity of the peptide moiety varies in each organism.[31] In *S. aureus* cell wall, the stem peptides (L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala) from adjacent glycan polymers are cross-linked via a pentaglycine bridge.[33] The cross-link is a peptide bond formed between the amino terminus of the pentaglycine branch on one stem peptide to the carboxylate end of the penultimate D-Ala residue on another. The cross-links impart strengths to PG as a protective layer. In *S. aureus* and other Gram-positive species, PG also serves as a scaffold for covalent attachment of cell surface oligosaccharide polymers such as wall teichoic acids (WTAs) and capsular polysaccharides, as well as a number of proteins.[34]

The biosynthesis of PG is a multi-step process. Much of our understanding of the reaction sequence in the pathway was revealed in conjunction with studies on penicillin's mechanism of

[c] Lysozyme, another significant discovery of Alexander Fleming, is a substance found in human body fluids that acts as a glycosidase to kill bacteria by hydrolyzing the bacterial cell wall.

action in bacteria in the 1950s-1970s. The following sections summarize the three stages of PG biosynthesis using *S. aureus* as the primary example, while many of the steps are conserved in all bacteria.[34]

1.2.1 Formation of the Park nucleotide

In 1949, Park J. T. made a curious but seminal observation that several related nucleotide species were strongly accumulated in *S. aureus* treated with a non-lethal concentration of penicillin.[35] The principal species, which was identified as uridine-5'-pyrophspho-*N*-acetyl-muramyl-L-alanyl-D-glutamyl-L-lysl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide), was later named the Park nucleotide (Figure 1.2), while the other species contain incomplete peptide moieties.[36-38] The accumulation of the Park nucleotide in *S. aureus* was not irreversible, since removal of penicillin from the culture led to its reduction. The Park nucleotide was also present at a low level in untreated *S. aureus* as well, thus excluding its possibility as a toxic byproduct due to penicillin treatment. Instead, the rapid accumulation of the Park nucleotide in penicillin-treated *S. aureus* reflected its high flux under normal conditions, suggesting its involvement in a major metabolic pathway in cell.[39] The striking structural similarity between the Park nucleotide and *S. aureus* PG led Strominger J. L. and coworkers in 1959 to proposes that the Park nucleotide is a biosynthetic precursor of the cell wall, and the accumulation of the Park nucleotide is a direct effect of penicillin inhibition.[33] They further established that the related nucleotides containing shorter peptides were intermediates in the formation of the Park nucleotide in *S. aureus*.[40-42]

It wasn't until the early 1990s that the individual enzymes involved in the formation of the Park nucleotide were characterized, owing to the ability to overexpress and purify candidate

proteins. The first committed step in the pathway is the conversion of UDP-GlcNAc to UDP-MurNAc by MurA and MurB enzymes in the cytoplasm. MurA transfers phosphoenolpyruvate (PEP) to UDP-GlcNAc, generating an intermediate that is further reduced by MurB reductase using NADPH.[43,44] MurA is the target of fosfomycin (fos), a natural product inhibitor that is a structural analogue of PEP, which can covalently bind to the active site cysteine residue in MurA.[45] In the subsequent steps, Mur ligases (MurC–F), which all belong to the ATP-dependent amide bond-forming enzyme family, catalyze the sequential attachment of L-Ala, D-isoGlu, L-Lys (or *meso*-diaminopimelic acid (*m*-DAP) in most Gram-negative bacteria) and D-Ala-D-Ala onto the C3-lactoyl moiety of UDP-MurNAc to yield UDP-MurNAc-pentapeptide precursor (the Park nucleotide). The D-Ala-D-Ala dipeptide is synthesized by the D-Ala-D-Ala ligase (Ddl), another specific ATP-dependent ligase, which condenses two molecules of D-Ala.[46] Vancomycin (vanco), an important glycopeptide antibiotic that is considered as a 'last-resort' antibiotic in the clinic, acts by specifically binding to the D-Ala-D-Ala termini in PG to kill bacteria. In vancomycin-resistant bacterial strains, MurF incorporates D-Ala-D-Lac[d] depsipeptide (a product of the Van ligase) into the Park nucleotide.[47-49] Strains that use altered peptide termini in PG in place of the normal peptide can effectively evade vancomycin sequestration.

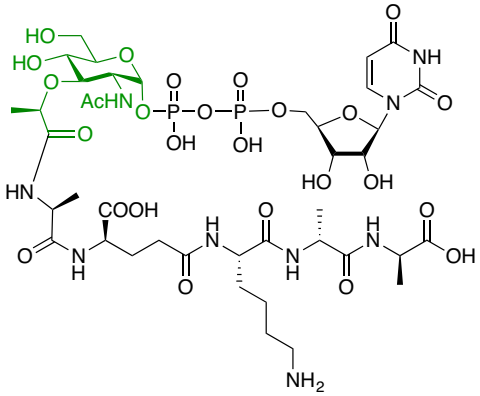

Figure 1.2. Structure of the Park nucleotide (UDP-MurNAc-pentapeptide) in *S. aureus*.

[d] Lac refers to a lactate residue.

1.2.2 Formation of the lipid-linked intermediates

The involvement of the Park nucleotide in bacterial cell wall synthesis was validated by several labs (1964) using *S. aureus* or *Micrococcus luteus* particulate enzymes *in vitro*.[41,50] The particulate enzymes, which were crude preparations of membrane proteins, could utilize the radioactive Park nucleotide and UDP-GlcNAc to yield an insoluble radiolabeled product that was lysozyme-sensitive. The product was characterized as a linear polymer made of alternating GlcNAc and MurNAc units, resembling the glycan backbone in bacterial cell wall.[49] Strominger and coworkers uncovered the complexity of the bacterial cell wall pathway when they analyzed *in vitro* reaction mixture on paper chromatography using isobutyric acid-ammonia eluent, which is the prototype of *in vitro* PG synthesis assay that is still prevalent today.[51,52] In the paper chromatogram, the radiolabeled polymeric PG product was immobile and retained at the origin, while the soluble nucleotide migrated slightly up from the baseline. However, a third fast-moving radioactive spot appeared near the top of the paper, which was identified as a mixture of two species, one containing MurNAc-pentapeptide, and the other containing MurNAc-pentapeptide and GlcNAc. The high mobility of both species on paper chromatography in the organic eluent implied they were lipid-linked, while their occurrence in the reaction was consistent with them being the reaction intermediates. These lipid-linked intermediates were quantitatively extracted from the reaction mixture using organic solvents of pyridinium acetate/n-butanol, while mild acid hydrolysis of the isolated intermediates yielded two soluble products, GlcNAc-MurNAc-pentapeptide and MurNAc-pentapeptide, where the composition of the pentapeptide was identical to that of the Park nucleotide.[51] In each intermediate, a pyrophosphate linkage was identified connecting the reducing end of the MurNAc unit to a lipid moiety. While the general structures of the intermediates, MurNAc-(pentapeptide)-P-P-lipid

(known as Lipid I) and GlcNAc-MurNAc-(pentapeptide)-P-P-lipid (known as Lipid II) were determined, elucidation of the nature of the lipid moiety was complicated by the low availability of these materials. Remarkably, Higashi *et al.* managed to scale up the particulate enzyme reaction by nearly 100,000-fold to achieve approximately 4 µmoles of the purified Lipid II[e] that permitted structural studies.[53] After removal of the soluble fragment on Lipid II by acid hydrolysis, the lipid moiety was purified and conclusively identified as undecaprenol (Und) by mass spectrometry. The Und lipid contains eight *cis*- , two *trans*-, and one terminal isoprene units, as characterized in nuclear magnetic resonance (NMR) studies.[53,54] The chemical structures of Lipid I and Lipid II intermediates are shown in Figure 1.3, while the reactions leading to their formation are summarized below:

UDP-MurNAc-pentapeptide + Und-P → UMP + MurNAc-(pentapeptide)-P-P-Und [Lipid I]

Lipid I + UDP-GlcNAc → UDP + GlcNAc-MurNAc-(pentapepitde)-P-P-Und [Lipid II]

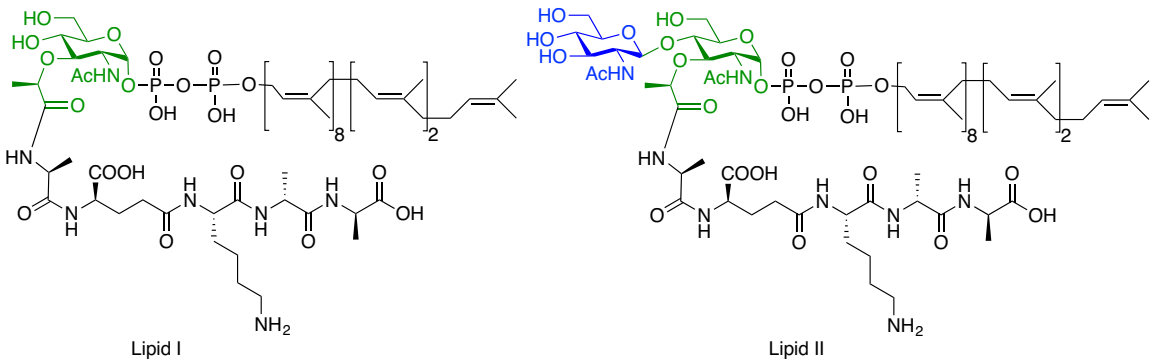

Figure 1.3. Structures of Lipid I and Lipid II intermediates identified in the cell-free system using the particulate enzymes from *S. aureus*.

The essential *mraY* and *murG* genes, which encode the enzymes in Lipid I and Lipid II synthesis, were identified in 1991.[55-58] In the first step, MraY, which belongs to the polyprenylphosphate *N*-acetyl hexosamine 1-phophate-transferase (PNPT) superfamily, transfers

[e] 4 µmoles of Lipid II is equivalent of 8 mg.

the phospho-MurNAc-pentapeptide moiety from the Park nucleotide to the Und-P acceptor in the cytoplasmic face of the membrane to form Lipid I.[59] Early work by Anderson *et al.* using particulate enzymes had demonstrated that Lipid I formation was reversible *in vitro*.[51] Unfortunately, the complex transmembrane architecture of MraY has posed a challenge to its enzymatic studies. In 2004, Bouhss *et al.* reported the first purification of the *Bacillus subtilis* (*B. subtilis*) MraY protein to homogeneity in milligram quantities, which opened ways for its biochemical characterizations.[60] Chung *et al.* (2013) recently solved the crystal structure of *Aquifex aeolicus* (*A. aeolicus*) MraY ($MraY_{aa}$), which represents the first structure of the PNPT protein superfamily.[61] In the $MraY_{aa}$ structure, a number of invariant residues were identified near the active site cleft, in which a Mg^{2+} was bound. Interestingly, a hydrophobic groove connected to the $MraY_{aa}$ active site was observed, which was proposed to be the binding site for Und-P substrate.[61] In addition, MraY demonstrates promiscuous substrate specificity to lipid lengths and configurations in the *in vitro* syntheses of Lipid I analogues.[62,63]

In the second reaction, the glycosyltransferase MurG catalyzes the addition of GlcNAc from UDP-GlcNAc donor to the C4 hydroxyl of Lipid I to yield Lipid II. Using synthetic Lipid I analogues containing variable lipid moieties, Walker and coworkers reconstituted MurG activity in 1998, and further established its intrinsic lipid preference (see Section 1.5).[64] MurG reaction proceeds via an ordered Bi-Bi mechanism in which UDP-GlcNAc binds first, prior to the formation of a ternary complex.[65] Consistent with the proposed mechanism, crystal structures of apo-MurG as well as MurG in complex with UDP-GlcNAc have been obtained.[66,67] Notably, in Lipid II formation, the α-anomeric configuration of UDP-GlcNAc is inverted to the β-(1-4)-glycosidic linkage in Lipid II.[64] In bacteria, MurG is believed to rapidly turn over Lipid I to Lipid II, based on the observation that Lipid II is always in excess of Lipid I in bacteria.[68]

In addition to the conserved MraY and MurG reactions, additional modifications on Lipid II occur in most Gram-positive bacteria. The complete *S. aureus* Lipid II structure (Figure 1.4) was deduced by Strominger and coworkers in the late 1960s, based on the observation that the linear PG product formed *in vitro* differs from the isolated *S. aureus* cell wall in two aspects: one is amidation on the α-carboxylate group of D-isoGlu, the other is the attachment of a pentaglycine branch to the ε-amino group of L-Lys in the pentapeptide.[69-71] The enzymes responsible for these additional modifications in *S. aureus* Lipid II have been revealed and characterized recently. Three enzymes, FemXAB, which belong to the non-ribosomal peptidyltransferase enzyme family, sequentially assemble the complete pentaglycine branch using glycyl-tRNA as donors, such that FemX adds the first, FemA adds the second and third, and FemB add the last two glycines.[71] Enzymatic studies of the Fem proteins showed that they are highly specific to the respective acceptor substrates (i.e. Lipid II for FemX, Gly-Lipid II for FemA and Gly_3-Lipid II for FemB).[72,73] The activity of each Fem protein does not require the presence of the other ones, but the formation of the complete pentaglycine branch is delayed *in vitro* when all three proteins are present, implying that competitive, non-productive binding occurs between non-cognate protein and acceptor pairs.[73] While FemX is essential, FemA and FemB are dispensable in *S. aureus* but are critical for resistance in methicillin-resistant *S. aureus* (MRSA) strains (see Chapter 1.4). Homologues of Fem proteins are present in other Gram-positive species for branch peptide formation. For instance, bppA1 and bppA2 transferases are responsible for the L-Ala-L-Ala branch *in Enterococcus faecalis* (*E. faecalis*), MurM transferases form the L-Ser-L-Ala (or L-Ala-L-Ala) branch in *Streptococcus pneumoniae* (*S. pneumoniae*).[72] On the other hand, Lipid II in Gram-negative bacteria commonly has an *m*-Dap at the third position of the stem peptide and does not have a branch peptide.[72]

Amidation of the α-D-isoGln in Lipid II is a common feature in *Staphylococci, Streptococci* and *Mycobacteria*, but is not observed in many Gram-negative bacteria.[72] In *S. aureus*, Lipid II amidation is catalyzed by two enzymes, a glutaminase GatD and an amidotransferase MurT, in an ATP- and ammonia-dependent reaction.[74] A decrease in the amidation content in PG in MRSA reduces methicillin resistance (see Chapter 1.4). In addition, bacterial PG amidation is shown to reduce the activation of host immune response, which may provide a survival advantage to bacteria during infection.[75,76] Nevertheless, the *in vivo* sequence of these additional modifications on *S. aureus* Lipid II (i.e. pentaglycine branch and amidation) is yet to be determined, since both FemXAB and GatT/MurT enzymes are capable of modifying Lipid II independently *in vitro*.[73,74]

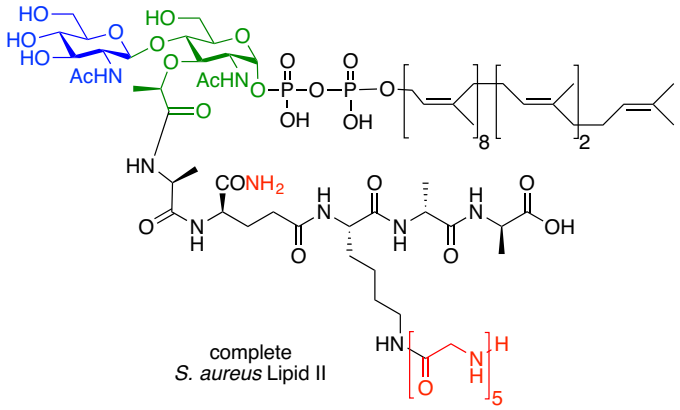

Figure 1.4. The complete Lipid II structure in *S. aureus.* The moieties in red indicate additional modifications on Lipid II precursor. The pentaglycine branch is unique to *S. aureus* Lipid II.

Around the time when the existence of lipid-linked intermediates (Lipid I and Lipid II) in *S. aureus* PG synthesis was first established in the mid-1960s, the involvement of lipid-linked oligosaccharide intermediates in the lipopolysaccharide (LPS) pathway in *Salmonella* was revealed as well.[77] Indeed, the use of Und lipid-linked substrates is a common strategy in the

biosynthesis of many bacterial cell surface polysaccharides,[f] such as LPS *O*-antigen, enterobacterial common antigen (ECA), WTA and capsular polysaccharide.[78] In eukaryotes, the dolicol-linked oligosaccharides are precursors in the *N*-linked protein glycosylation in endoplasmic reticulum (ER).[79] The membrane-bound lipid carriers provide a mean to translocate hydrophilic precursors across the hydrophobic cytoplasmic membrane for processes in the extracellular face of the membrane (or in the ER lumen in the case of the eukaryotic dolicol pathway). Thus, the involvement of a translocase or flippase in the membrane was envisioned to facilitate the energetically unfavorable translocation process. However, the translocation step in bacterial PG biosynthesis is poorly understood and has remained elusive, until recently, several candidates of Lipid II flippase in bacteria are proposed.

In 2008, Ruiz N. first proposed MurJ (formerly MviN), an essential inner membrane protein in *E. coli*, is the Lipid II flippase.[80] MurJ is conserved in PG-producing endosymbiotic bacteria that have lost most of their non-essential genes, but it is absent in bacteria that lack PG. Depletion of MurJ in *E. coli* results in an accumulation of PG precursors and induces cell rounding and blebbing, reminiscent to the morphological observations of penicillin-treated bacteria by Gardner and others in the 1940s that were indicative of PG synthesis inhibition.[16] In addition, MurJ belongs to the multidrug/oligo-saccharidyl-lipid/polysaccharide (MOP) exporter superfamily, which also encompasses other polysaccharide transporters (PSTs) such as WzxE, the flippase for ECA lipid-linked precursors, and WzxB, the LPS *O*-antigen flippase, as well as the oligosasaccharidyl-lipid flippase (OLF) Rtf1p, the proposed flippase in the dolicol-pathway.[81] Since its discovery, many lines of evidence have given credence to MurJ as the Lipid II flippase. For instance, MurJ is predicted to have a hydrophilic cavity based on topological studies (such cavities are present in many multidrug exporters in the MOP family); chemical

[f] *Mycobacteria* are known to use decaprenyl-lipid carriers instead.

blockage of this cavity in MurJ leads to accumulation of Lipid II in the inner membrane in *E. coli*.[82-84] In addition, the *murJ* orthologue, *ygtP* is found to be essential in *S. aureus* and *S. pneumonia*,[85] whereas *B. subtilis* possesses multiple *ygtP* homologues that are non-essential, yet an unrelated *amJ* gene can substitute the Lipid II flipping activity in *B. subtilis*.[86] Controversially, another flippase candidate, FtsW has been proposed in *E. coli*, which is an essential protein for cell division that belongs to the shape, elongation, division and sporulation (SEDS) family.[87] FtsW is shown to translocate Lipid II as well as phospholipids *in vitro* using vesicle assays, but its role as a flippase has not been validated *in vivo*.[88]

1.2.3 Peptidoglycan (PG) assembly

After translocation of Lipid II to the outer leaflet of the cytoplasmic membrane, it is polymerized by peptidoglycan glycosyltransferases (PGTs) to form long glycan strands that contain β-(1,4)-glycosidic linkages between the C4 of GlcNAc and the C1 of MurNAc units. The formation of linear PG polymers has been demonstrated in early works on PG synthesis.[41,50] However, unlike the nascent PG product *in vitro*, the isolated *S. aureus* cell wall is a highly cross-linked material, in which the amino end of the pentaglycine branch on one stem peptide is attached to the carboxylate group of the penultimate D-Ala residue on another. Thus, it was interpreted that nascent PG must undergo a transpeptidation reaction in the final stage of PG assembly, the enzymes that carry out this step are named transpeptidases (TPs).[89] The major steps in *S. aureus* PG biosynthesis are shown in Figure 1.5. However, due to the difficulties in obtaining the complete *S. aureus* Lipid II (see Chapter 1.5), transpeptidation in *S. aureus* has not been demonstrated *in vitro*.

With the recognition of the complete reaction sequence in PG biosynthesis, penicillin's mechanism of action was visited by Tipper and Strominger in 1965.[89] Since penicillin did not inhibit the formation of nascent PG in the particulate cell-free reactions, its involvement in the final transpeptidation reaction was highly likely. Based on the structural similarities between the 6-APA core of penicillin (which is in essence an acylated cyclic dipeptide of L-Cys and D-Val) and the D-Ala-D-Ala terminus in PG, Tipper and Strominger proposed that penicillin inhibits transpeptidation by acting as a substrate analogue.[89,90] It was postulated that in the transpeptidation step, a TP recognizes the D-Ala-D-Ala terminus of the stem peptide and cleaves the terminal D-Ala residue to form an acyl-enzyme intermediate, then the incoming pentaglycine branch attacks the covalent intermediate to form a cross-link, thus freeing the TP enzyme (Figure 1.6). Transpeptidation does not require any ATP input, consistent with a lack of ATP in the extracytoplasmic space in bacteria. Penicillin contains a highly reactive amide bond in the beta-lactam ring that could be viewed as the equivalence of the amide bond in D-Ala-D-Ala terminus. Thus, when the TP enzyme attacks the beta-lactam ring in penicillin instead, a penicilloyl-enzyme adduct is yielded, inactivating the TP enzyme (Figure 1.6). Consistent with the proposed mechanism of penicillin, uncross-linked nascent PG polymers were significantly accumulated in penicillin-treated *S. aureus*.[90]

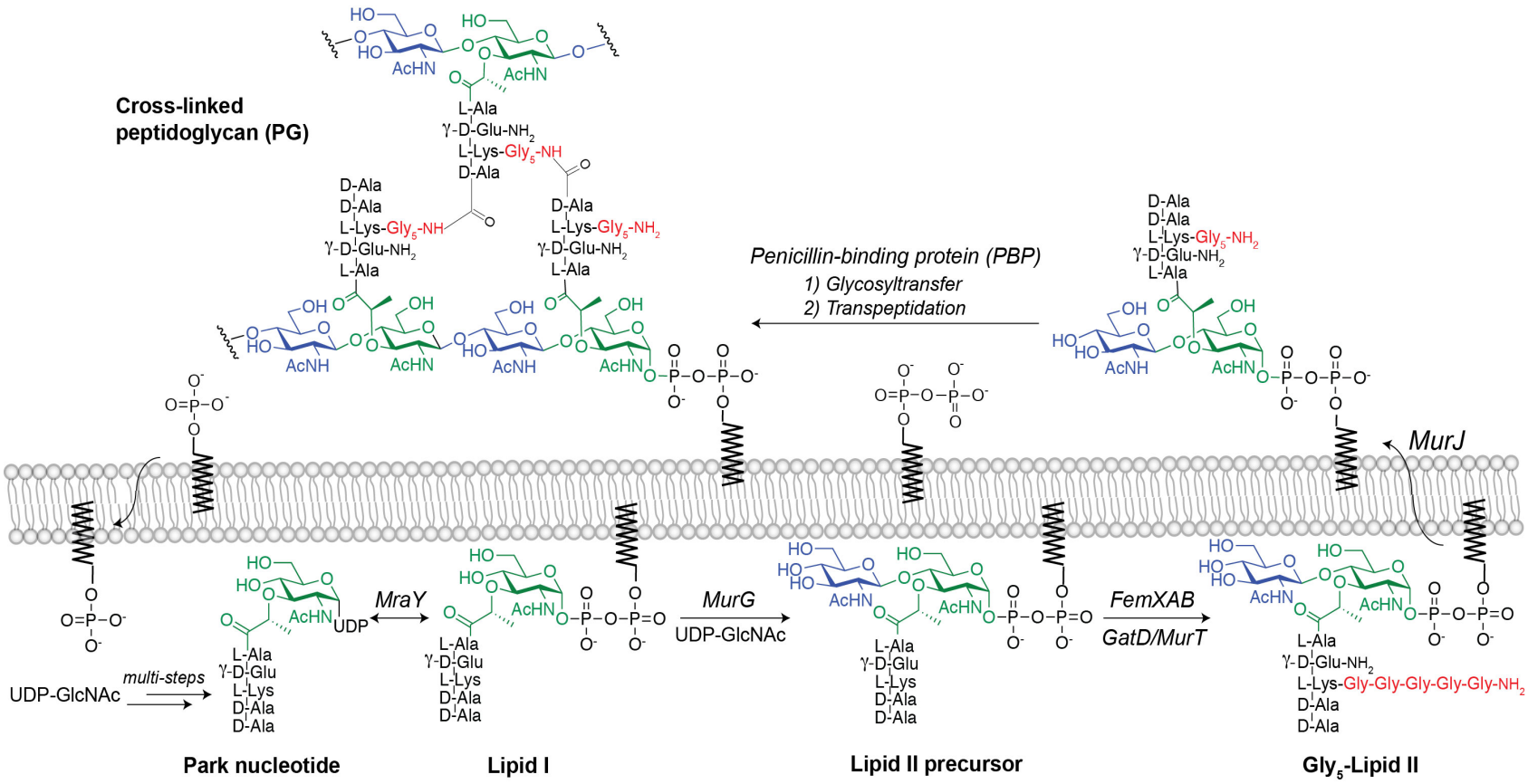

Figure 1.5. Schematic showing peptidoglycan (PG) biosynthesis in *S. aureus*. The process occurs in three main stages: 1) formation of the Park nucleotide in the cytoplasm, 2) formation of lipid-linked intermediates (i.e. Lipid I and Lipid II) in the inner leaflet of the cytoplasmic membrane, and 3) PG assembly at the outside of the membrane in which Lipid II is polymerized and cross-linked.

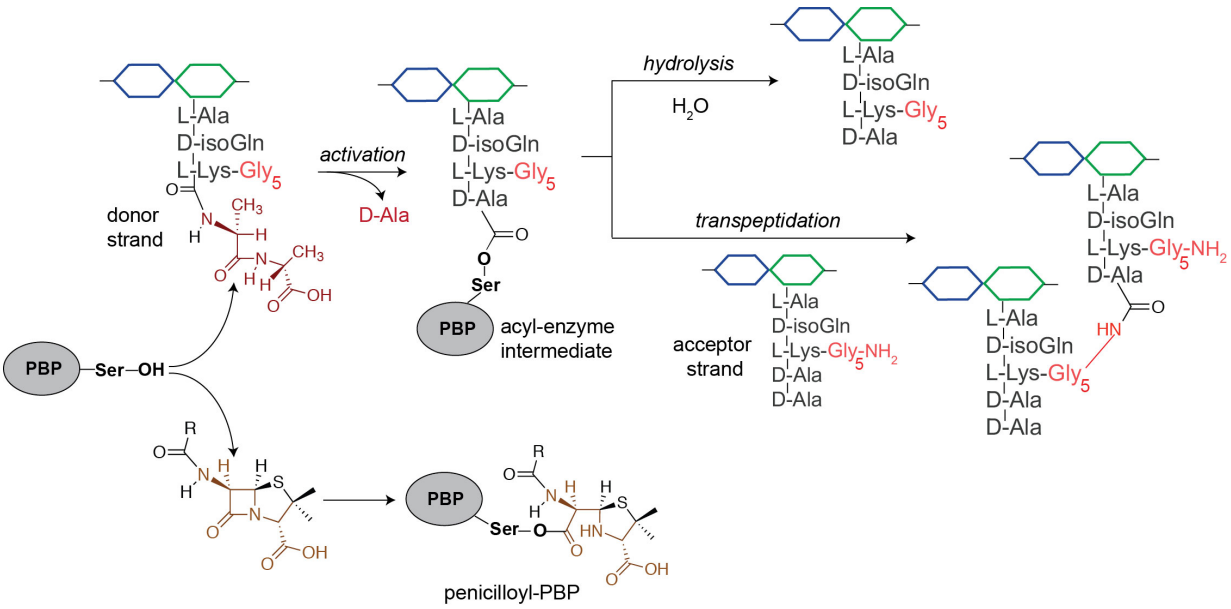

Figure 1.6. The proposed mechanisms of transpeptidation (and hydrolysis) by PBPs and penicillin inhibition. In this model, the beta-lactam ring in penicillin (highlighted in brown) resembles the conformation of the D-Ala-D-Ala terminus in a stem pentapeptide (in crimson color). In PG assembly, PBPs reacts with the D-Ala-D-Ala terminus on a donor strand to form an acyl-enzyme intermediate, with the concomitant release of the terminal D-Ala. The intermediate is attacked by the pentaglycine branch on an acceptor strands to form a cross-link (transpeptidation). Alternatively, in the case of carboxypeptidase, the acyl-enzyme intermediate is resolved by water. Penicillin inhibits PG biosynthesis by covalently acylating the PBP active site.

In efforts to identify the cellular target(s) of penicillin in bacteria, the old observation of penicillin binding to bacterial membrane was examined in greater detail in the 1960s-1970s. The intricacy of the biosynthetic enzymes in the final step of bacterial PG assembly was then revealed.[91,92] Multiple penicillin-binding proteins (PBPs), defined as proteins that covalently interact with penicillin, were detected when the radiolabeled penicillin was incubated with either whole cells or particulate membranes from bacteria. Radiolabeled PBPs, which could be separated on sodium dodecyl sulfate (SDS) gel electrophoresis and detected by fluorography, were numbered in order of descending molecular weights in each organism.[93-97] In *S. aureus*, there are four native PBPs, PBP1-4, while MRSA has acquired an additional one, PBP2a (see Chapter 1.4).[93,97] PBPs can also be classified into high-molecular weight (HMW) Class A and

Class B PBPs, and low molecular weight (LMW) PBPs. HMW Class A PBPs, such as *S. aureus* PBP2, contain both TP and PGT domains, while HMW Class B PBPs, such as *S. aureus* PBP1, 2a and 3, contain TP domain and an N-terminal domain of unknown function. In *S. aureus*, PBP4 is the only LMW PBP.[98] In addition, *S. aureus* also contains two nonessential monofunctional PGTs, SgtA and SgtB.[99]

1.3 The cellular roles of *S. aureus* penicillin-binding proteins (PBPs)

The detection of multiple PBPs in bacteria unequivocally validated the proposed inhibition mechanism of penicillin, in which a stable penicilloyl-enzyme adduct is formed.[89] However, the observation also raised several questions: which of the PBPs are the lethal targets of penicillin? How do the activities of multiple PBPs differ in bacteria and what are their cellular roles? It was soon realized that PBPs in a given organism display widely different sensitivities to penicillin and other beta-lactams.[100] In general, LMW PBPs appear less sensitive to penicillin, and are capable of releasing the bound penicillin either spontaneously or by exogenous hydroxylamine nucleophiles, while HMW PBPs are relatively more sensitive. The binding affinity of a non-radiolabeled beta-lactam for PBPs is usually assessed in a competition assay, in which the concentration of the beta-lactam required to block the binding of labeled penicillin is determined.[95,96] However, quantitative analysis of binding affinity depends on many variables such as temperature, incubation time, and protein concentrations of the assay.[100,101] Beta-lactams that selectively bind to individual PBPs in *S. aureus* have been identified. For instance, as reported by Okonog *et al.* and shown in Figure 1.7*,* cloxacillin, ceftizoxime, cephalexin and cefoxitin are specific binders to *S. aureus* PBP1, PBP2,[g] PBP3, and PBP4 respectively at

[g] The binding of ceftizoxime to PBP2 was not complete at concentrations used in the experiment (ref. 102).

concentrations that do not affect other PBPs.[102] Together with genetic approaches, studies using beta-lactams as chemical probes to inhibit specific PBPs have shed light on the physiological roles of individual PBPs in bacteria.

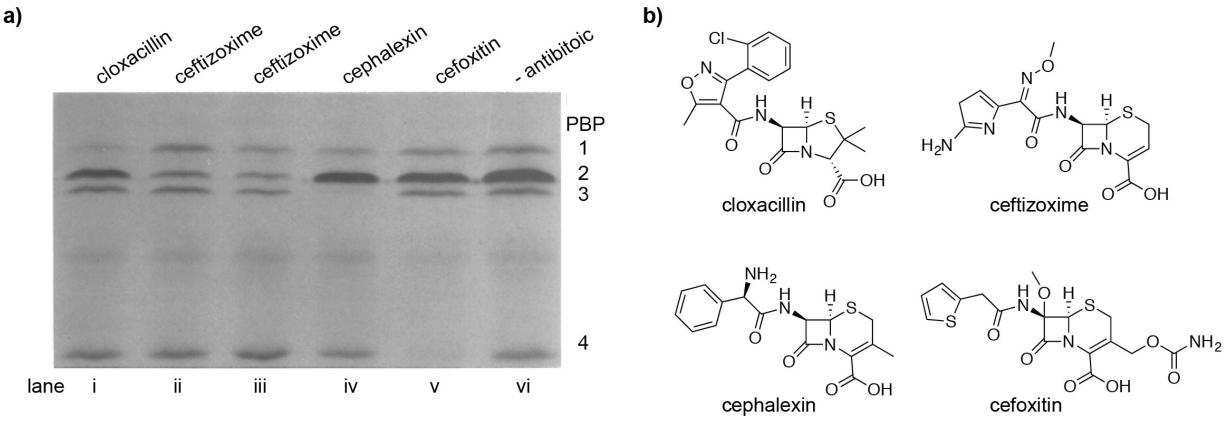

Figure 1.7. Beta-lactams show selective binding to *S. aureus* PBPs. Figure a) is modified from ref.102. Briefly, growing *S. aureus* cells were incubated with cloxacillin (0.06 µg/mL) (i), ceftizoxime (0.1, 0.6 µg/mL) (ii-iii), cephalexin (0.08 µg/mL) (iv), and cefoxitin (0.05 µg/mL) (v), or in the absence of an antibiotic (vi). The cells were disrupted and incubated with ^{14}C-penicillin G, and were subjected to SDS-PAGE and fluorography. The concentrations of the beta-lactams in use were above the *in vitro* inhibitory concentrations. b) Structures of beta-lactams used are shown.

S. aureus PBP1 is an essential HMW Class B PBP,[103] whose function was initially elucidated by Okonog *et al.* using cloxacillin as a probe.[102] Selective inhibition of PBP1 by cloxacillin caused *S. aureus* cells to swell and become irregularly shaped, while manifesting aberrant septum formation. Similar abnormal septa formation was observed in a PBP1-deficient MRSA mutant, in which the expression of PBP1 was under the control of an inducible promoter.[104] Thus, *S. aureus* PBP1 is proposed to play an essential role in regulating cell division. Subsequently, the specific function of the PBP1 TP domain was addressed by Pereira *et al.,* using a *S. aureus* mutant strain in which the TP catalytic residue of PBP1 was specifically mutated.[105] The strain was viable but showed separation defects after a few rounds of normal cell divisions, and demonstrated decrease in PG cross-linking. Taken together, the TP activity of *S.*

aureus PBP1 is not essential for viability, but is required for cell separation at the end of division.[105] PBP1 was proposed to be part of a regulatory circuit that ensures proper PG synthesis and degradation in *S. aureus*, although the underlying mechanism remains elusive.[105]

S. aureus PBP2 is a HMW Class A PBP that has both PGT and TP domains. Using genetic strategies to selectively inactivate the catalytic residue in PBP2 PGT or TP domain, Pinho *et al.* investigated the essentiality of each domain in PBP2.[106,107] The TP domain, which is essential in methicillin-susceptible *S. aureus* (MSSA), becomes dispensable in MRSA in the presence of an additional PBP2a (see Chapter 1.4).[106] On the other hand, the inactivation of PBP2 PGT domain does not normally affect *S. aureus* viability, but strongly sensitizes MRSA to beta-lactams.[107] Taken together, these observations imply that PBP2 is the major TP for PG cross-linking in *S. aureus*, but it can be replaced by PBP2a in MRSA; whereas PBP2 PGT activity is required to cooperate with PBP2a to maintain PG synthesis in MRSA in the face of beta-lactams. However, when grown in the absence of beta-lactams, *S. aureus* PBP2 PGT activity can be replaced by the monofunctional PGT, SgtB.[108h] *S. aureus* PBP2 is the lethal target of moenomycin, a natural product PGT inhibitor, since a resistant mutant that bears a point mutation in the PBP2 PGT active site cleft has been isolated. The mutant displays an increase in moenomycin resistance by 25-fold.[109] In addition, the TP domain of PBP2 was thought to be responsible for its localization at the division septum in *S. aureus* by interacting with the D-Ala-D-Ala termini in PG, since either acylation of PBP2 TP domain by oxacillin or modification of the PG termini by D-cycloserine and vancomycin results in delocalization of PBP2.[110] In

[h] *S. aureus* has two monofunctional PGTs, SgtA and SgtB, both of which are nonessential when PBP2 is present. However, only SgtB can compensate the PGT activity when PBP2 PGT domain is mutated (ref. 108). The transglycosylase activity of SgtB has been reconstituted in vitro (ref. 171), but the activity of SgtA has not been reconstituted in vitro.

addition, a spontaneous ceftizoxime-resistant mutant of *S. aureus* was identified with a point mutation in the PBP2 TP domain, suggesting that PBP2 is the lethal target of ceftizoxime.[111]

S. aureus PBP3 is another HMW Class B PBP, which can be deleted without causing detectable change in *S. aureus* cell growth or PG composition,[112] contrary to the previous interpretation that PBP3 is essential in the competition binding assays.[113] However, treating the *S. aureus* ΔPBP3 mutant with a sub-lethal concentration of methicillin results in abnormal cell shape and size, indicating that inhibition of other PBPs in the absence of PBP3 is deleterious to *S. aureus* cell morphology.[112]

Aside from the HMW PBPs in *S. aureus*, PBP4 is the only LMW PBP. Because of its reversibility in penicillin binding by hydroxylamine, *S. aureus* PBP4 was purified and characterized by Kozarich and Strominger in 1978.[114] PBP4 did not only hydrolyze the terminal D-Ala in the Ac_2-L-Lys-D-Ala-D-Ala tripeptide, indicating its carboxypeptidase activity, but also incorporated Gly or hydroxylamine acceptors in place of the terminal D-Ala, demonstrating TP activity. Under prolonged incubation with a high amount of penicillin, *S. aureus* PBP4 also demonstrated penicillinase activity *in vitro*, although it was not considered physiologically relevant.[114] Its dual activities as a CP and TP *in vitro* were quite perplexing. Like other LMW PBPs, *S. aureus* PBP4 is non-essential for cell viability, thus it is not a lethal target of penicillin.[115] However, S. aureus ΔPBP4 mutant manifests a drastic reduction in the level of PG cross-linking, suggesting the cellular role of PBP4 in PG secondary cross-linking.[116] *S. aureus* PBP4 and PBP2 are proposed to cooperate *in vivo* to produce the highly cross-linked cell wall in *S. aureus*.[111] The inactivation of PBP4 in *S. aureus* causes intermediate vancomycin resistance in several laboratory and clinical isolates,[117,118] while an overproduction of PBP4 in certain *S. aureus* strains leads to an increase in beta-lactam resistance.[119-121] However, the effect of *S.*

aureus PBP4 on antibiotic resistance can be variable depending on the strain background.[122] In the community-acquired MRSA (CA-MRSA) strains, PBP4 is essential for the expression of beta-lactam resistance.[123] Inactivation of PBP4 strongly sensitized the CA-MRSA to oxacillin and nafcillin. Cefoxitin, a selective inhibitor of *S. aureus* PBP4, strongly synergizes with oxacillin at killing CA-MRSA, which may provide a potential therapeutic strategy against CA-MRSA infections. Furthermore, WTAs are proposed to ensure proper localization of PBP4 at the division septum in *S. aureus*,[124] although recently, it is shown that *S. aureus* PBP4 can display extraseptal cross-linking activity as well.[125]

Table 1.1. Classification of PBPs in *S. aureus.* PBP1-4 are native PBPs in *S. aureus*, PBP2a is an acquired PBP in MRSA.

S. aureus PBPs	Class	Cellular roles	Essentiality	Biochemical characterization
PBP1	HMW (Class B)	Cell division; PG cross-linking	Yes (but not the TP domain)	No
PBP2	HMW (Class A)	Major PBP in PG polymerization and cross-linking	PGT not essential if SgtB is present; TP is essential, but can be replaced by PBP2a in MRSA	Only PGT[126], not TP
PBP2a (MRSA)	HMW (Class B)	Confers resistance	Requires PBP2 PGT domain to function	No
PBP3	HMW (Class B)	Unclear	Not essential	No
PBP4	LMW PBP	Secondary PG cross-linking	Not essential, but is required for resistance in CA-MRSA	CP/ TP/ beta-lactamase

A combination of genetic, microscopic and chemical approaches has been useful to dissect the cellular roles of individual PBPs in *S. aureus*. However, their TP activities have not

been demonstrated *in vitro* (Table 1.1). This thesis focuses on the development of tools to study TP activities of two *S. aureus* PBPs (PBP4 and PBP2). Biochemical characterizations of bacterial PBPs will not only contribute to our understanding of their redundant and interdependent activities, but also may facilitate the discovery of potential antibiotics.

1.4 Resistance in methicillin-resistant *S. aureus* (MRSA)

Since the first introduction of penicillin in the 1940s, which marks the dawn of an antibiotic era, antibiotics have been frequently misused and overused. As a result, antibiotic resistance in bacteria has emerged rapidly and has become alarming in recent years. CDC estimated that at least two million people are infected with resistant bacteria each year and at least 23,000 people die as a direct result of such infections in the United States.[127] At the current rate, it is projected that 10 million deaths worldwide will be attributed to resistant bacterial infections each year by 2050.[128] Among the most threatening resistant pathogens, MRSA is one that is widespread and causes the most number of deaths each year.[129] Although new antibiotics have been developed to target MRSA, including the fourth and fifth generations of cephalosporins, resistance against these new drugs has also emerged.[130] In order to develop new strategies to combat MRSA, understanding the mechanism of resistance in MRSA is of paramount importance.

1.4.1 An acquired resistant PBP, PBP2a in MRSA

A defining characteristic of MRSA is the presence of staphylococcal cassette chromosome *mec* (*SCCmec*) in the chromosome.[i] *SCCmec* is a mobile genetic element about 40-

[i] In some cases, *S. aureus* strains may have other genetic determinants for low-level resistance in the absence of the *mecA* gene.

60 kb in length and includes a *mecA* gene.[131] The transcription of *mecA* is normally repressed by the regulatory elements *mecI* and *mecRI* that are also part of the *SCCmec*, its repression can be alleviated by certain beta-lactams.[132] However, many clinical MRSA isolates have mutations in the regulatory elements such that *mecA* is constitutely expressed.[133] *SCCmec* is proposed to originated from *Staphylococcus sciuri* (which contains homologous genes), but the mechanism of acquisition is not clear.[134] The MRSA *mecA* gene encodes PBP2a, a HMW Class B PBP, which displays low affinity to penicillin and other beta-lactams in the competition binding assay.[135,136] PBP2a is assumed to continue PG cross-linking in MRSA under conditions that native PBPs are inhibited by beta-lactams. However, MRSA manifests a decrease in PG cross-linking when grown in the presence of beta-lactams.[107] This observation implies that PBP2a activity cannot entirely compensate for other native PBPs but is enough for MRSA survival in the face of beta-lactam inhibition.

Kinetic binding studies have shed light on the intrinsic resistance of PBP2a against beta-lactams. *S. aureus* PBP2a displays a slow acylation rate (k_2) and a low affinity to most beta-lactams (high K_d) (Figure 1.8a), both of which work in concert to contribute to its intrinsic resistance.[137,138] The poor acylation rate (k_2) of *S. aureus* PBP2a by beta-lactams can be rationalized by its having a distorted TP active site that needs to undergo an unfavorable conformational change for acylation, as perceived in the apo- and acylated-PBP2a crystal structures.[139] Cephalosporins, which possess extended substituents on the beta-lactam core, can make significant Van der Waal interactions in the narrow active site groove of PBP2a. Such non-covalent interactions are thought to contribute to cephalosporins' enhanced affinity to PBP2a (reduced K_d) and hence potent antibacterial activities. In light of this, non-covalent inhibitors that exhibit strong affinity to PBP2a may represent promising anti-MRSA strategies.[139]

S. aureus PBP2a's intrinsic resistance to acylation offers MRSA an advantage to beta-lactam inhibition. However, it must be overcome during transpeptidation, in which PBP2a presumably forms an acyl-enzyme intermediate with the stem peptide in PG. Mobashery and coworkers established that the presence of cell wall fragments can stimulate beta-lactam acylation of PBP2a *in vitro*.[140] In the recent crystal structures of *S. aureus* PBP2a by the same group, ligands (i.e. muramic acid, cell wall fragment or ceftaroline) bound in a location 60 Å distal from the TP active site in PBP2a were identified (Figure 1.8b).[141] This observation led to the proposal of an allosteric regulation in *S. aureus* PBP2a TP activity: binding of a ligand in the allosteric domain in PBP2a may allow the TP active site to open up for catalysis.[141] The allosteric model awaits validation in a TP activity assay of *S. aureus* PBP2a. Nevertheless, such allostery may have been exploited by ceftaroline, the recently approved fifth-generation cephalosporin, to re-sensitize *S. aureus* PBP2a to beta-lactam inhibition.[142,143] Ceftaroline's interaction with PBP2a allosteric domain in addition to acylation of the TP active site suggest an unprecedented inhibitory mechanism.[141] Binding of ceftaroline in the allosteric site is thought to trigger a series of salt bridge interactions in PBP2a that spans from the allosteric to the TP domain, predisposing the TP active site for acylation. In the clinical isolates of ceftaroline-resistant MRSA, several point mutations in the allosteric site of PBP2a have been identified.[144] These mutations were shown to disrupt the interaction network in the allosteric site responsible for the stimulation of the TP active site, leading to resistance.[144] However, such mutations are expected to disturb PBP2a TP activity as well. The TP activities of PBP2a and its variants are yet to be demonstrated. In addition, a similar allosteric domain is present in the crystal structure of *Enterococcus faecium* PBP5, but whether allosteric regulation applies is yet to be determined.[141]

a)

$$E + I \underset{K_d}{\rightleftharpoons} EI \xrightarrow{k_2} E\text{-}I \xrightarrow{k_3} E + P$$

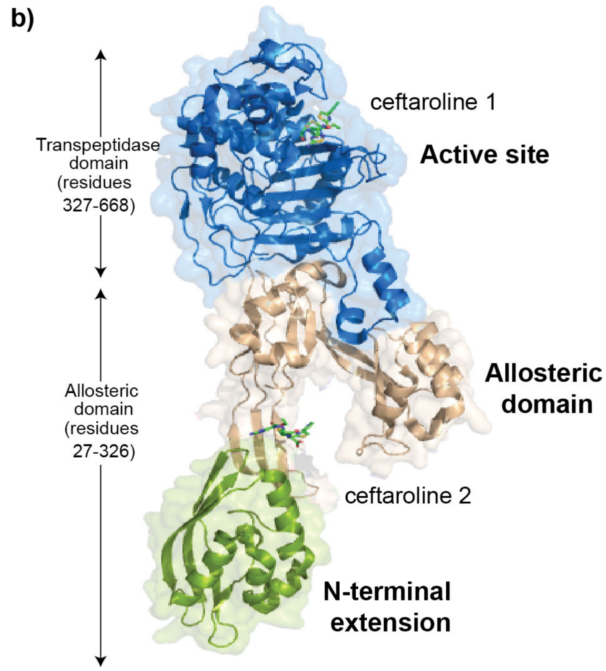

Figure 1.8. An allosteric regulation of *S. aureus* PBP2a is proposed. a) The equation representing the chemical reaction of beta-lactam acylation of PBP and release. E represents PBP, EI is the non-covalent pre-acylation complex, E-I is the covalent acyl enzyme species, and P denotes the product of hydrolysis of the beta-lactam antibiotic. K_d is the dissociaton constant for the formation of the non-covalent complex, k_2 is the first-order rate constant for the acylation reaction, and k_3 is the first-order rate constant for deacylation. b) Ribbon representation of crystal structure of *S. aureus* PBP2a bound with ceftarolines both in the allosteric site and TP active site. Figure modified from ref. 141.

1.4.2 Factors influencing methicillin resistance

Although *mecA* is the primary resistant gene in MRSA, it alone is not enough to confer resistance and additional genes are required for the full expression of resistance. Many of these genes, named *fem* (for f̲actors e̲ssential for m̲ethicillin) or *aux* (auxiliary) factors, were identified in transposon mutagenesis studies of MRSA mutants that display reduced methicillin resistance. In particular, the *femA* and *femB* inactivation produced the most significant reduction in methicillin resistance in MRSA.[145,146] As mentioned in Chapter 1.2.2, FemA and FemB are

enzymes responsible for pentaglycine branch formation in Lipid II in *S. aureus* PG pathway. *S. aureus* Δ*femA* and Δ*femB* mutants both demonstrate a drastic reduction in cross-linking and glycine contents in PG, with monoglycine branch predominantly found in the former and triglycine branch in the latter.[147,148] It is hypothesized that PBP2a has a stringent substrate requirement for the complete pentaglycine branch in PG cross-linking, while shorter branches in Δ*femA* and Δ*femB* mutants abolish its TP activity and re-sensitize MRSA to beta-lactams.[149] Therefore, targeting FemA or FemB protein in combination with beta-lactams may provide a novel therapeutic strategy against MRSA. Reportedly, a small molecule FemA inhibitor, cyslabdan that effectively potentiates beta-lactams against MRSA has been recently identified.[150,151] The substrate specificity of PBP2a needs to be validated in order to establish a mechanistic basis for such re-sensitization strategy in MRSA.

In addition, several genes that are either directly or indirectly involved in the formation of native *S. aureus* Lipid II were found to affect beta-lactam resistance in MRSA. For instance, inactivation of the glutamine synthetase repressor, glnR, which reduces the amidation on D-isoGln on PG, results in lowering of beta-lactam resistance in MRSA.[152] Moreover, genes involved in other aspects of the cell surface can also contribute to beta-lactam resistance in MRSA, such as, *fmtA,* a predicted membrane protein with sequence homology to PBPs that is recently identified as a D-alanyl esterase of teichoic acids,[153,154] as well as a large number of genes including *tarO*, *tarA*, *tarD*, *tarS* and *ltaS* in the WTA pathway and in the lipoteichoic acid (LTA) pathway.[155-157] Global regulators, *agr* and *sar,* which control the expression of many cell surface proteins were identified as well.[158] Since many of these factors play important roles in *S. aureus* cell division, growth and autolysis, their inactivation also sensitizes MSSA to methicillin instead of directly influencing the activity of PBP2a in MRSA *per se.*[159]

1.4.3 Chemical genetic approaches to combat MRSA

The diverse *fem* and *aux* factors identified in transposon mutagenesis studies represent potential points of intervention. Inhibition of these targets by small molecules may phenocopy the effects of their genetic inactivation to re-sensitize MRSA against beta-lactams. The combination of such inhibitors with beta-lactams holds great promise as a novel therapeutic strategy against MRSA. In recent years, many groups have performed high-throughput screening of chemical libraries to identity compounds that can potentiate beta-lactam activity in MRSA.[150,160-162] As mentioned earlier, a potential FemA inhibitor, cyslabdan, was identified as an imipeniem potentiator.[150] Similarly, Huber *et al.* reported two related compounds CDFI and DMPI that act to potentiate imipenem activity in *S. aureus* COL. The inhibitors were proposed to target SAV1754 that is the proposed Lipid II flippase in *S. aureus*,[162] representing a new class of inhibitors in *S. aureus*. A recent study demonstrates that a collateral application of beta-lactams (carbapenem/penicillin/beta-lactamase) is effective at killing MRSA *in vitro* and clearing MRSA infections in a mouse model.[163] In conclusion, chemical genetic screenings prove useful in identifying novel beta-lactam potentiators as well as establishing new cellular targets of inhibition in MRSA.

1.5 Lipid II is required for enzymatic studies of PBPs

Although bacterial PBPs have been identified as the cellular targets of beta-lactams since the 1970s, characterizations of their enzymatic activities (i.e. PGT and TP) have lagged behind for several decades, due to challenges in purifying these membrane-bound proteins and in obtaining the complex Lipid II substrates. The cell-free system using particulate enzymes for Lipid II production was laborious and inefficient; the direct isolation of Lipid II from bacteria

was challenging given their natural low abundance in bacteria.[68] It has been estimated that there are no more than 700 copies of Lipid I molecules and 2000 copies Lipid II molecules per *E. coli* cell;[68] while the numbers are generally higher in Gram-positive bacteria, Lipid II molecules still represents less than 1% of the total phospholipids in the membrane.[164] Since the early 2000s, advance in synthetic and enzymatic preparations of Lipid II and analogues[64,165-168] accompanied with the development of specific activity assays[168-170] have enabled mechanistic studies of bacterial PBPs. Many PBPs from different organisms have been overexpressed and purified to homogeneity in full-length and/or soluble forms, whose activities were characterized *in vitro* using defined substrates.[126,171-175] The following sections will discuss current methods for Lipid II preparations, and key understanding of PGT and TP catalysis.

1.5.1 Current approaches of Lipid II preparation

In 1998, Walker and coworkers synthesized a citronellol-Lipid I (C10) analogue using a convergent approach to assemble the three building blocks of the molecule: the MurNAc derivative, the pentapeptide, and the lipid tail.[64] In particular, the pyrophosphate linkage that connects the reducing end of the MurNAc residue to the lipid was constructed using the diphenyl chlorophosphate activation method. This work represents the first chemical synthesis of a lipid-linked PG precursor. Using synthetic Lipid I analogues, the authors investigated the enzymatic activity of MurG *in vitro*, which laid the foundation for a facile chemoenzymatic strategy to prepare Lipid II. Subsequently, utilizing the 1,1'-carbonyldiimidazole (CDI) coupling method that is tolerant of the allylic phosphate ester in pyrophosphate formation, Kahne, Walker and coworkers (2001) prepared the natural undecaprenyl-Lipid I and a number of Lipid I derivatives of varying lipid lengths and double bond geometries, and converted to them to the corresponding

Lipid II derivatives using MurG.[168] The accessibility to various Lipid II derivatives of different lipid moieties shed light on the substrate preference of *E. coli* PGTs for the very first time (see Chapter 1.5.2). In the same and following years, two groups reported the total chemical syntheses of Lipid II, both utilizing a CDI-catalyzed coupling of undecapenyl phosphate to the appropriately protected GlcNAc-MurNAc-pentapeptide moiety at the late stage of the multi-step synthetic scheme.[165,167] While milligram quantities of Lipid II could be obtained from synthesis, the overall yield was quite low; for instance, only 0.7% overall yield was obtained after a 12-step synthesis, as reported by Schwartz *et al.*[165] In addition, several fluorescent Lipid II derivatives have been synthesized as well.[176-178] Most of the synthetic Lipid II contains the L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala pentapeptide, which resembles the common Lipid II precursor in most Gram-positive bacteria, can be used as a substrate by bacterial PGTs *in vitro*; they are not suitable for studying bacterial TPs since they lack the canonical peptide branch.[179]

Recognizing the formation of the allylic pyrophosphate in Lipid I and Lipid II via the phophorimidazolidate intermediate is rather sluggish, Tsukamoto and Kahne improved the reaction rate by adding an efficient catalyst, *N*-methylimidazolium chloride, which significantly reduced the reaction time from a few days to 12 hours.[180] In the new synthetic route to make Lipid I (Figure 1.9), Tsukamoto and Kahne first introduced the pyrophosphate bond to form the MurNAc-PP-lipid, which is the conserved moiety among all bacterial Lipid I, followed by the addition of the peptide. Lipid I could then be quantitatively converted to Lipid II by MurG chemoenzyamtically.[64] Given that the peptide portion of Lipid II is varied in bacteria, the new route that introduces the peptide late in the synthesis allows easier preparations of diverse Lipid II. Via this route, the native *E. coli* and *B. subtilis* Lipid II substrates, which contain an *m*-Dap and an amidated *m*-Dap residue at the third position of the stem peptide respectively, were

synthesized and used for characterizations of the corresponding bacterial PBPs.[179,181,182] In addition, the more complicated Lipid IV substrate, which represents an elongated product of two Lipid II molecules, has been chemically synthesized to probe the activity of PGTs.[183,184]

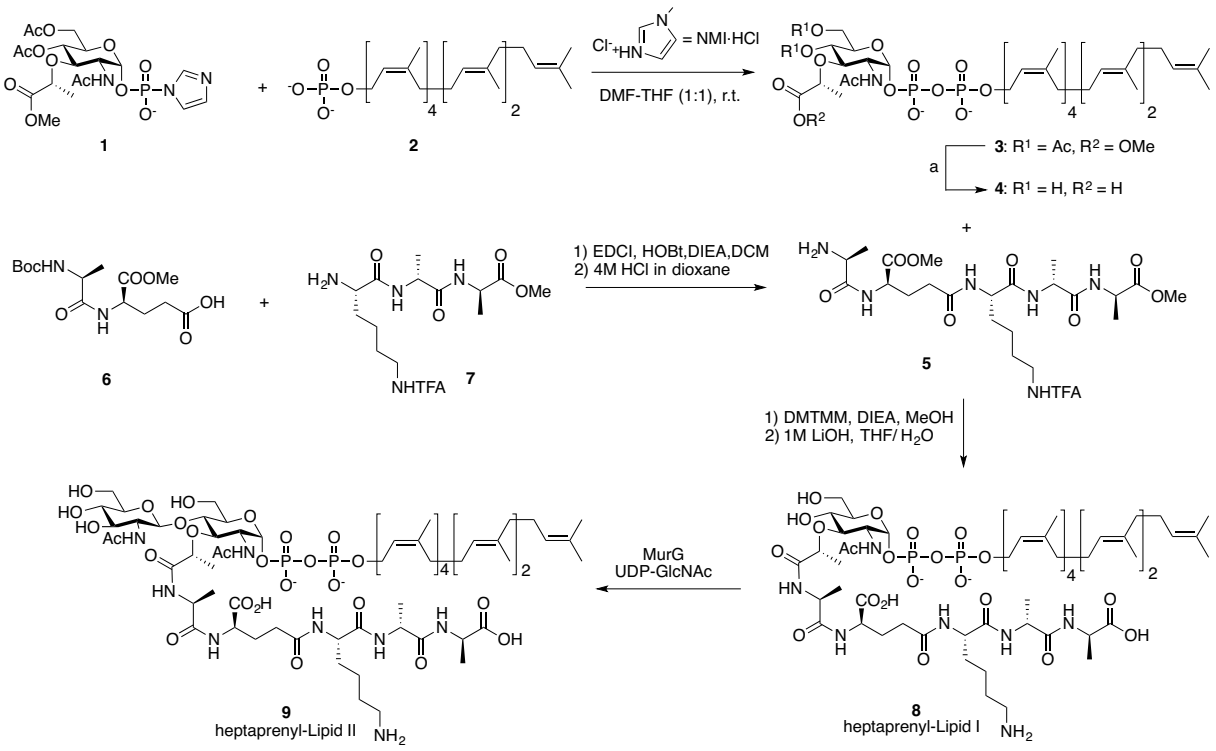

Figure 1.9. The synthetic route to make Lipid I by Tsukamoto and Kahne (ref. 175). In this route, the conserved moiety MurNAc-PP-lipid precursor (**3**) is prepared first, prior to the coupling of the peptide portion, which is variable in different bacteria.

In addition to the above mentioned synthetic routes, another well-established approach to prepare Lipid I and Lipid II molecules involves biochemical reconstitutions. This approach has its origin in the particulate enzyme reactions for studying PG synthesis, by which Lipid I and Lipid II intermediates were initially identified (see Chapter 1.2.2).[51] In such reactions, the Park nucleotide, UDP-GlcNAc and the particulate enzymes, which contain membrane proteins MraY and MurG as well as the Und-P precursors, were incubated. However, the low availability of

Und-P in the membrane has rendered Lipid II production rather inefficient.[68] Breukink *et al.* (2003) overcame the technical hurdle by supplying exogenous polyisoprenol phosphate to the reaction, improving the yield of the reaction by at least a 100-fold.[63] In particularly, the substrates used in the reaction are isolated from various natural sources.[185] For instance, the Park nucleotide is usually extracted from bacteria that are treated with vancomycin, which allows cellular accumulation of the Park nucleotide.[186] Depending on the bacteria source, the peptide composition in the isolated Park nucleotides may vary.[j] The polyisoprenoids can be isolated from plant leaves and are phosphorylated by undecaprenol kinase (UK) *in vitro*.[185] In addition, MraY exhibits broad substrate specificity to a large number of isoprenyl phosphates, including the dolichol-type isoprenyl phosphate, yielding a number of Lipid I variants.[62,63] Although relatively large amount of Lipid II (50-100 mg) can be obtained with ease from biochemical productions, preparations of native Lipid II containing the canonical branch peptide (in Gram-positive bacteria) are significantly more challenging since multiple additional enzymes are required for reconstitution. Remarkably, the *in vitro* assembly of the pentaglycine branch in *S. aureus* Lipid II has been achieved by Schneider T *et al.,* involving a complex reaction mixture that includes purified FemXAB proteins, glycyl-tRNA synthease, staphylococcal tRNA pool, as well as purified Lipid II precursors obtained from the particulate enzyme system.[73] The small amount of pentaglycine-Lipid II formed in the reaction could be conveniently detected on thin layer chromatography (TLC), but its isolation and purification was not possible, thus obviating its utility as a substrate for studying *S. aureus* PBPs TP activities. Similarity, the GatD/MurT enzymes responsible for amidation in *S. aureus* Lipid II have been recently reconstituted *in vitro*, but the amidated Lipid II product could not be achieved in useful quantities.[74,175] The amidated

[j] *S. aureus* is used for extraction of the L-Lys containing Park nucleotide, and *Bacillus cere*us is a good source for the *m*-Dap containing Park nucleotide.

Lipid II in *S. penumoniae* has been obtained and shown to be important for efficient PG cross-linking.[174,175] Conceivably, the exquisite features of *S. aureus* Lipid II are pre-requisites for studying *S. aureus* PBPs TP activities. However, obtaining the native *S. aureus* Lipid II containing both the pentaglycine branch and amidation is deemed the bottleneck to such studies.

1.5.2 Mechanistic insights into peptidoglycan glycosyltransferase (PGT) activity

The accessibility to various Lipid II analogues has enabled enzymatic studies of bacterial PGTs.[k] Kahne, Walker and coworkers (2001) investigated the substrate preference of *E. coli* PBP1b (a HMW Class A PBP) using various synthetic Lipid II substrates.[168] It was found that the presence of a *cis* double bond at the allylic position in Lipid II is critical for PGT activity. In addition, a shorter heptaprenyl Lipid II analogue was identified as a better substrate than the natural undecaprenyl Lipid II for *E. coli* PBP1b activity *in vitro*, presumably due to its less tendency to aggregate.[168,187] In general, the PGT activity of a bifunctional PBP does not depend on its TP activity, since the PGT domain can be isolated, overexpressed and purified, and demonstrate comparable activities as the full-length protein.[188] Following the development of a high-resolution SDS-PAGE assay that can resolve long glycan polymers according to their lengths, Walker and coworkers showed that bacterial PGTs are processive enzymes that catalyze multiple rounds of polymerization without releasing the glycan strands.[169] Interestingly, PGTs from different organisms produce glycan polymers of distinct lengths *in vitro*,[171] implying a possible intrinsic control of product length. By preparing a number of short PG oligomers that are blocked with an galactose (Gal) at the non-reducing end, Perlstein *et al.* established that the nascent PG glycan chains elongate with the addition of Lipid II acceptors to the reducing end of

[k] The absence of native branch peptide in Lipid II substrate does not affect PGT activity. Therefore, Lys-Lipid II is suitable for studying PGT activity *in vitro*.

the growing polymer, solving a fundamental question regarding PGT activities.[170] The ability to differentiate donor and acceptor substrates in PG polymerization led to the recognition that PGTs demonstrate different requirements for substrate lipid lengths at the two (donor and acceptor) sites.[189] While PGTs are promiscuous to the length and geometry of the acceptor lipid, they require a lipid of at least 20 carbons in the donor site. The donor lipid is also the major determinant of the processivity of polymerization. In addition, time course analysis of the *in vitro* PGT reaction revealed a prolonged lag phase, which was attributed to the slow initial coupling of two Lipid II molecules in the donor and acceptor sites. This rate-limiting step in PG polymerization could be bypassed by pre-incubating PGTs with a Gal-Lipid IV analogue, which mimics the initial coupling product and serves as the 'donor-only' substrate.[190] Thus, it was proposed that PGTs may undergo different conformational states. A glycan strand containing at least four sugars bound in the donor site may facilitate the reorganization of PGT to an activated conformation for PG elongation. While the structure of PGT in complex with Gal-Lipid IV still awaits, a number of crystal structures of bacterial PGTs that are either in the apo-forms or bound with inhibitor (moenomycin) or Lipid II analogue are available, providing important structural information of PGT activities.[188,191-194]

The crystal structures of bacterial PGTs reveal a unique globular domain that is composed of mainly alpha helices representing a structural class of glycosyltransferase.[191] Despite little sequence homology, PGTs bear some structural resemblance to the bacteriophage λ-lysozyme, the lytic glycosidase that cleaves the β-1,4-glycosidic bond between the C1 of MurNAc and the C4 of GlcNAc residues in PG with a catalytic glutamic acid residue. Sequence alignments show that bacterial PGTs contain five conserved motifs containing a number of invariant residues with the glutamic acid in the first motif being the catalytic residue.[188] The

putative glutamic acid residue in the PGT domain is proposed to abstract the C4-OH proton of GlcNAc in Lipid II, which then attacks the reducing C1 position on MurNAc in the donor strand, forming a new β-1,4-glycosidic bond in the PG product.[188] The reaction is facilitated by another conserved residue in the PGT active site that stabilizes the released undecaprenol pyrophosphate. Mutagenesis studies have confirmed the essentiality of these residues for PGT activity.[188]

Several interesting features regarding the PGT active site are revealed in the crystal structures of PGTs bound with moenomycin, a natural product inhibitor of PGTs.[188,191-193] Moenomcyin binds in an extended conformation that makes a large hydrogen-bonding network with PGT, which is proposed to be the donor substrate binding site.[192] The donor site in PGT appears more positively charged and is capable of forming numerous hydrogen-bond interactions, providing strong binding energy to translocate the elongated PG polymer.[194] Crystal structure of *S. aureus* PBP2 in complex with moenomycin showed a dramatic conformational rearrangement in its jaw subdomain when compared to the apo-enzyme structure;[191] whereas moenomycin binding did not elicit noticeable changes in the PGT domain of *A. aerolicus* PBP1a and in *S. aureus* SgtB.[188,192-194] Such conformational difference in PGTs may reflect their structural dynamics during PG synthesis. A crystal structure of *S. aureus* SgtB in complex with a Lipid II analogue was reported recently, in which the polar portion of the analogue was observed in the PGT acceptor site, providing a new snapshot of the PG polymerization process[194]. Going forward, structural information on how a growing glycan strand interacts with the donor site of PGT is attractive to study.

1.5.3 Existing knowledge on PBP transpeptidase (TP) activities

In contrast to the wealth of information on bacterial PGT activities, mechanistic studies of TPs are limited, since the specific Lipid II substrates are required for studying TP activities of PBPs from different organisms.[I] The *E. coli* Lipid II, which contains an *m*-Dap at the third position of the stem peptide, has been prepared either by biochemical production or chemical synthesis and used for characterization of *E. coli* HMW PBPs, PBP1a and 1b.[172,173,179] The TP activity of a HMW PBP is coupled to its PGT activity, and cannot act alone.[195] A general strategy is used to study HMW PBP TP activity: purified *E. coli* PBP1A or PBP1B is incubated with *m*-Dap Lipid II, the product is then digested by a glycosidase to disaccharide units, which could be separated and analyzed on liquid chromatography (LC). The presence of cross-linked muropeptides indicates successful transpeptidation by the PBP.[172,173,179] In addition, *E. coli* PBPs are capable of attaching nascent PG polymers formed *in vitro* to the isolated sacculi via transpeptidation.[172,173] Most existing knowledge on TP activities come from HMW Class A PBPs, while TP activities of Class B PBPs have not been demonstrated. Banzhaf *et al.* has inferred the activity of *E. coli* PBP2 (a Class B PBP) based on its stimulatory effects on *E. coli* PBP1a cross-linking activity *in vitro*, but the direct evidence of PBP2 TP activity is lacking.[196] The activities of PBPs are thought to cooperate during PG synthesis in bacteria.[107,111,196] For instance, *S. aureus* PBP2 and PBP4 are thought to cooperate together to produce the highly cross-linked PG.[110] However, the inaccessibility of native Lipid II substrates in useful quantities has hampered mechanistic studies of PBPs TP activities in Gram-positive bacteria.

Using synthetic Lys-Lipid II that cannot act as an acceptor in the transpeptidation reaction, Lupoli *et al.* (2011) showed that *E. coli* PBP1a could incorporate D-amino acid into the terminal stem peptide position in PG.[195] The D-amino acid incorporation by TP can be perceived

[I] The tripeptide substrate has been used to study the TP activities of LMW PBPs, but not HMW PBPs.

as the reverse of the activation step in transpeptidation, in which the terminal D-Ala is released (Figure 1.6). However, the tolerance of PBP to a wide range of D-amino acids as acceptors *in vitro* is unexpected.[195] On the other hand, it has been observed that a variety of bacteria can uptake and incorporate exogenous D-amino acids into PG during growth, although the positions and mechanisms of incorporation vary.[197,198] In *E. coli*, exogenous D-amino acids are exclusively incorporated into the fourth position of the stem peptide in PG *in vivo* and the L,D-transpeptidases (Ldts) are responsible for such incorporation.[198] In *B. subtilis*, HMW PBPs mediate D-amino acid incorporation into PG *in vivo*.[198,199] In *S. aureus*, exogenous D-amino acids are incorporated into the terminal peptide position in PG,[199] but the mechanism of incorporation was not clear at the time when we initiated the project described in Chapter Two. In addition, several intracellular pathogens, including *Chlamydia trachomatis* and *Listera monocytogenes*, incorporate exogenous D-amino acid into PG via the cytoplasmic Ddl and MurF ligases.[200-202] Many research groups have harnessed the power of D-amino acid incorporation to develop fluorescent D-amino acid probes, which have provided new insights into PG synthesis in various organisms.[125,199-203]

1.6 Perspectives

Although penicillin-binding proteins (PBPs) have been exploited by beta-lactams for over seventy years, it is remarkable that the transpeptidase (TP) activities of PBPs in many bacteria, including *S. aureus*, have not been directly demonstrated. In particular, the activities of PBPs in *S. aureus* form the basis of resistance in methicillin-resistant *S. aureus* (MRSA), a major threat to public health. This thesis discusses our efforts on studying the TP activities of major PBPs in *S. aureus*. A major challenge is the preparation of useful quantities of native *S. aureus* Lipid II

substrate. Chapter Two describes the studies of *S. aureus* PBP4 and the development of a simple and sensitive assay to evaluate the cellular pools of Lipid II in bacteria. This observation has led to a facile method to accumulate and extract Lipid II from *S. aureus* culture (Chapter Three). Although our work is focused on *S. aureus*, the approach can be generalized to other bacterial organisms as well. Lastly, we have reconstituted the TP activity of *S. aureus* PBP2 and assessed its inhibition by a panel of beta-lactam antibiotics (Chapter Four). The biochemical tools prepared in this work should be useful for studies of resistance in MRSA and facilitate the development of better therapeutic strategies against MRSA.

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Chapter Two: Exploiting the transpeptidase activity of PBP4 to detect peptidoglycan precursors in cells and to elucidate the mechanism of antibiotics that target cell wall biosynthesis

Work presented in this chapter was adapted from

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2.1 Introduction

This chapter describes work to characterize the activity of a low-molecular weight (LMW) penicillin-binding protein (PBP) in *Stapylococcus arureus*, PBP4. Unlike previously characterized LMW PBPs in the literature that act as carboxypeptidases (CPs), we showed that *S. aureus* PBP4 has transpeptidase activity. Moreover, unlike previously characterized high-molecular weight (HMW) PBPs, we demonstrated that PBP4 can use Lipid II as a substrate rather than depending on the concomitant peptidoglycan glycosyltransferase (PGT) activity. We exploited the unexpected transpeptidase activity of *S. aureus* PBP4 to develop a facile assay for detection of cellular Lipid II in bacteria. We demonstrated that the assay is useful for studying the mechanisms of action for cell wall-targeting antibiotics and identified conditions that accumulate Lipid II in bacteria, laying the foundation for work described in Chapter Three.

2.1.1 *S. aureus* PBP4, a peculiar PBP

Bacterial growth and division require coordinations between peptidoglycan (PG) assembly and degradation.[1] PBPs, the major players in the final stages of PG biosynthesis, are involved in both processes.[2,3] The TP domain in HMW PBPs is responsible for peptide cross-linking between adjacent glycan strands to rigidify PG; LMW PBPs, most of which are also known as D, D-carboxypeptidases (CPs)[a], mediate the removal of the terminal D-Ala residue in the stem peptide in PG (Figure 2.1a).[4] Since a stem tetrapeptide can no longer be activated by TPs for cross-linking, LMW PBPs are thought to regulate the degree of PG cross-linking in bacteria.[5] While LMW PBPs are not essential for cell viability many still play important cellular functions in bacteria such as governing cell morphology and contributing to antibiotic

[a] A small number of LMW PBPs are endopeptidases (EPs), which catalyze the cleavage of an internal amide bond in the stem peptide in PG.

resistance.[4] As mentioned in Chapter 1.2.3, the name penicillin-binding protein reflects the fact these proteins were identified in radioactive penicillin binding studies. Sequence alignment reveals that PBPs contain three conserved signature motifs: SXXK, [S/Y]X[N/C] and [K/H][T/S]G, where the underlined serine is the catalytic residue.[6] These motifs adopt a strikingly similar conformation in all PBPs. In both TP and CP reactions, the first step involves the catalytic serine attacking the amide bond between D-Ala-D-Ala terminus of a stem peptide to form an acyl-enzyme intermediate with the concomitant release of the terminal D-Ala. Subsequently, the covalent intermediate is resolved by a nucleophile, the identity of which varies in TP and CP reaction. In the case of a CP, the nucleophile is water, and a tetrapeptide product is formed.[7] In the case of a TP, the nucleophile is the terminal amine from a stem peptide on an adjacent PG glycan polymer, and a cross-linked PG product is formed (Figure 2.1a).[8-14]

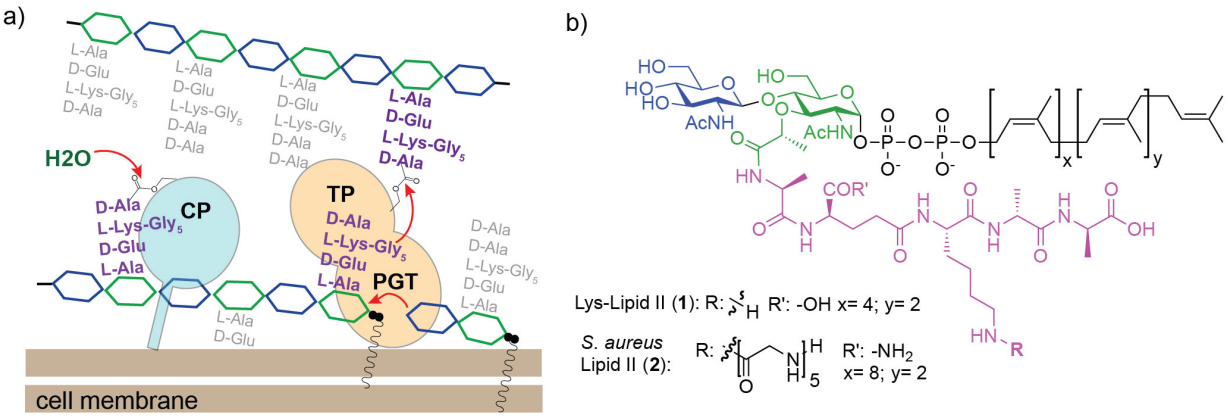

Figure 2.1. Schematic of carboxypeptidase (CP) and transpeptidase (TP) activities in *S. aureus* PG assembly (a) and Lipid II substrates (b).

PBP4 is the sole LMW PBP in *S. aureus*, which has been previously annotated as a CP based on sequence alignment to other characterized LMW PBPs in different organisms.[6] Like many LMW PBPs, *S. aureus* PBP4 has a predicted transmembrane helix at the C-terminus. *S.*

aureus PBP4 has two structural domains: an N-terminal domain with a beta-lactam/transpeptidase fold which contains the conserved motifs at the active site, and a C-terminal domain that is composed of mainly beta-sheets.[15] While the functional role of the C-terminal domain remains to be elucidated, it is also present in several other PBP4-like proteins such as *E. faecalis* D,D-carboxypeptiase, and *E. coli* PBP5.[16]

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PBP4_SAUR      68-DTKWNPASMTKLMTMYLT-85 133-QITVSNSSNAAALILA-148 250-MSLPGTDGLKTGSSDTA-266
PBP5*_BSUB     53-HQKRRIASITKIMTAVLA-70 109-YGLMLRSGNDAAVAIA-124 211-MLYPYSTGGKTGYTKLA-227
PBP5_PAER      57-DQRLPPASLTKLMTAYIA-74 118-HGIIIQSGNDASVALA-133 217-----TVDGLKTGHTDEA-233
PBP5_ECOL      66-DVRRDPASLTKMMTSYVI-63 133-RGINLQSGNDACVAMA-148 233----LNVDGIKTGHTDKA-249
PBP6_ECOL      59-DEKLDPASLTKIMTSYVV-75 126-KGVIIQSGNDACIALA-141 226----LNVDGMKTGTTAGA-242
                          *
```

Figure 2.2. Protein sequence alignments of representative LMW PBPs. Signature motifs are highlighted in red, the asterisk indicate the predicated catalytic serine residue. Abbreviations for each organism are as follows: *S. aureus* (SAUR), *B. subtilis* (BSUB), *P. aeruginosa* (PAER) and *E. coli* (ECOL). Alignment is done using Clustal Omega, based on work by Massova and Mobashery (1998).

As mentioned in Chapter 1.3, the biochemical activities of *S. aureus* PBP4 were investigated by Kozarich and Strominger in 1978 initially using a tripeptide substrate (Ac_2-L-Lys-D-Ala-D-Ala), which mimics the terminal portion of the stem peptide in Lipid II (Figure 2.1b).[17] Unlike most other LMW PBPs that behave solely as CPs, *S. aureus* PBP4 demonstrates CP, TP and beta-lactamase activity *in vitro*.[17] In the presence of a high concentration of the acceptor, such as glycine or hydroxylamine, the hydrolysis of the tripeptide was suppressed with the concomitant formation of the transpeptidation product. A new peptide was formed in which the terminal D-Ala was replaced by the acceptor. It was pointed out that the weaker nucleophile, glycine, was a better acceptor than hydroxylamine suggesting that *S. aureus* PBP4 recognized glycine as a preferred acceptor for transpeptidation. In addition, *S. aureus* PBP4 also demonstrated penicillinase activity upon incubation with penicillin. The ability of *S. aureus* PBP4 to carry out multiple reactions was intriguing. However, kinetic studies revealed that the

tripeptide substrate has a high K_m (100 mM),[17] suggesting it may not be an optimal substrate for analyzing *S. aureus* PBP4 activity *in vitro*. In addition, analysis of *S. aureus* PG reveals that over 90% of the stem peptides are cross-linked, with more than 50% being involved in high degree cross-linking, while no uncross-linked tetrapeptide stems are identified.[2,18] Genetic inactivation of *S. aureus* PBP4 leads to a drastic reduction in the higher levels of PG cross-linking, suggesting PBP4's physiological role in secondary PG cross-linking.[19] Taken together these studies did not resolve the physiological activities of PBP4.

To better understand the activity of *S. aureus* PBP4, we set out to reconstitute its activity using synthetic Lys-Lipid II as a substrate, which closely resembles native *S. aureus* Lipid II (Figure 2.1b). In this chapter, we showed that PBP4 has transpeptidase activity *in vitro*, which is distinct from other LMW PBPs that act as CPs solely. We next showed PBP4, the primary transpeptidase in *S. aureus* is responsible for fluorescent D-amino acid incorporation in PG *in vivo*. Upon investigation of its substrate specificity, we revealed a unique transpeptidase activity of PBP4: it can mediate D-amino acid exchange in Lipid II. *S. aurueus* PBP4 is the first transpeptidase demonstrated to activate Lipid II.

2.1.2 Challenges with studying the cellular pools of Lipid II in bacteria

The cellular pool of Lipid II in bacteria is a poorly addressed question, primarily due to the lack of a facile analysis method.[20] Scattered reports have addressed the number of Lipid I and II molecules in *E. coli* and have relied on intensive and laborious protocols.[21-23] In such studies, the cellular PG precursors were first radiolabeled by feeding the auxotrophic *E. coli* strain with radioactive *m*-Dap, followed by organic extraction of the lipid intermediates. The isolated radiolabeled lipid intermediates were then hydrolyzed with mild acid to release the

corresponding muropeptides, whose relative amount was compared to UDP-MurNAc-pentapeptide (the Park nucleotide) on HPLC. It was estimated that the ratio of the Park nucleotide: Lipid I: Lipid II in *E. coli* is approximately 300: 1: 3 or 140: 1: 3.[24,25] The ratios of Lipid II to Lipid I was found to be constant during the cell cycle.[24] Since the amount of the Park nucleotide is approximately 100,000 molecules per cell, there would be no more than 700 molecules of Lipid I and 1000 to 2000 molecules of Lipid II per cell in *E. coli*.[21]

In general, Gram-positive bacteria appear to have more Lipid II than Gram-negative bacteria.[22-26] In several Gram-positive bacteria, antibiotics (that act as binders to lipid-linked peptidoglycan intermediate) have been used to estimate the pool levels of lipid intermediates, where the number of antibiotics bound to cell membrane was taken as the number of Lipid II present.[20] For instance, binding of ramoplanin to *S. aureus* cells estimated that 50,000 molecules of lipid intermediates are present per cell.[27b] The amount of precursors estimated in such studies can vary considerably depending on the antibiotic and the bacteria.[28] Overall, Lipid II content accounts less than 1% of total phospholipids in bacteria.[29] The naturally low abundance of the lipid-linked PG precursors in bacteria renders them challenging to account for. Despite the fact that Lipid I and Lipid II are key intermediates in PG synthesis, how their pool levels change in bacteria under various pharmacological and genetic conditions has not been studied.

We exploited the unexpected transpeptidase activity of *S. aureus* PBP4 to develop a facile method to detect the cellular level of Lipid II in bacteria,[30] addressing a longstanding challenge in the field (see Chapter 2.6). We demonstrated that the assay is useful for elucidating the cellular mechanism of action for cell wall-targeting antibiotics. In a separate demonstration of the utility of these tools, we elucidated the mechanism of action of the antibiotic lysobactin.

[b] Ramoplanin is a lipoglycodepsipeptide that binds to Lipid II substrate to prevent PG polymerization.

We also identified conditions that lead to Lipid II accumulation in bacteria, which are important for studies in Chapter Three.

2.2 Purification and characterization of *S. aureus* PBP4

S. aureus PBP4 gene was cloned into an expression vector for heterologous overexpression in the *E. coli* host. Two C-terminally His_6-tagged PBP4 constructs were prepared initially.[c] One is a full-length construct (Mw is 48 kD); the other is a [Y21-Q383] truncated version that lacks the predicted N-terminal signal sequence and the C-terminal transmemrbane segment (Mw is 42 kD). Initial overexpression tests showed that the full-length PBP4 did not overexpress, but the truncated PBP4 protein was soluble and overexpressed well. The C-terminal transmembrane helix, a common feature in most LMW PBPs is likely to complicate the expression and localization of the full-length PBP4 construct. Therefore, we focused on the PBP4[Y21-Q383]-His_6 construct for purification and characterization, which will be denoted as PBP4 throughout the chapter. The soluble PBP4 construct was purified from the cytosolic fraction in *E. coli* by Ni-NTA (nitrilotriacetic acid) affinity column chromatography. PBP4 was purified to homogeneity after one step purification as observed on SDS-PAGE gel (Figure 2.3). The yield of purification is approximately 10 mg/L culture. A small amount of detergent (0.1% reduced triton-X) was added in the elution and storage buffer of PBP4 protein. Notably, the high concentration of imidazole in the elution buffer did not affect PBP4 stability and activity.

The Bocillin-FL binding assay was used to establish that the active site of PBP4 was properly folded.[31] In this assay, PBP4 was pre-incubated with various concentrations of non-labeled penicillin G (PenG), followed by the addition of a saturating amount of Bocillin-FL (Bo-FL), a fluorescent penicillin derivative to the reaction mixture. As shown in Figure 2.3, PenG

[c] A former postdoctoral researcher, Yuriy Rebets, prepared the constructs.

inhibited Bo-FL labeling of PBP4 in a dose-dependent manner, implying that active site of PBP4 is properly folded. However, such competition binding assays do not directly monitor activity of the PBPs, which will be discussed in more detail in Chapter Four.

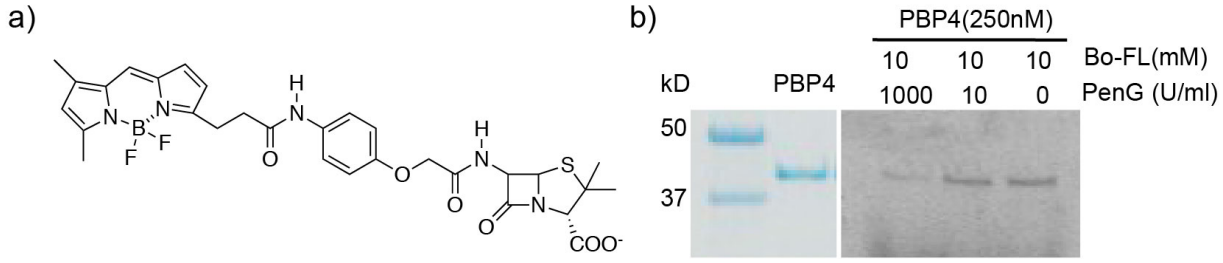

Figure 2.3. *S. aureus* PBP4 is purified to homogeneity. a) Structure of Bocillin-FL (Bo-FL), a fluorescent derivative of penicillin. b) Coomassie stained SDS-PAGE gel of purified PBP4 (left) and fluorescent image of Bo-FL-bound PBP4 in the presence of varying concentrations of penicillin (right). 1667 units (U) of penicillin equals to 1 mg.

2.3 Assessment of *S. aureus* PBP4 *in vitro* activity

To investigate the *in vitro* activity of *S. aureus* PBP4, we used synthetic Lipid II, Lys-Lipid II (Figure 2.1b).[32] As mentioned earlier, the Ac_2-L-Lys-D-Ala-D-Ala tripeptide has been used primarily to study LMW PBPs, including *S. aureus* PBP4;[15,17] however, the tripeptide substrate only contains a small portion of the Lipid II, which may affect the enzymatic activity.[d] In contrast, Lys-Lipid II shares the general lipid-linked disaccharide peptide structure with native *S. aureus* Lipid II except for a few distinctions. Lys-Lipid II lacks the pentaglycine branch peptide and contains a D-iso-Glu instead of D-isoGln at the second position of the stem peptide. Lys-Lipid II also has a shorter heptaprenyl lipid (C35) instead of the natural undecaprenyl lipid (C55). It has been demonstrated that heptaprenyl Lipid II analogues is a better substrate for PGT activity *in vitro*, due to its reduced tendency to aggregate.[33] The absence of pentaglycine branch

[d] The turnover number of PBP4 transpeptidase activity using tripeptide substrate was 20 min^{-1} (ref. 17).

in Lys-Lipid II for transpeptidation was overcome by the addition of exogenous oligoglycines as acceptors in the reaction (see below).

To evaluate the activity of *S. aureus* PBP4, we adopted a liquid chromatography-mass spectrometry (LC/MS) assay that was previously used to study HMW PBPs in *E. coli* and *B. subtilis*.[12,13] In this assay, PG polymer formed *in vitro* was digested with mutanolysin, a glycosidase into disaccharide muropeptides. The muropeptides were then treated with $NaBH_4$ to resolve the anomers at the reducing end of MurNAc prior to LC/MS analysis. Since mutanolysin can only digest PG polymer containing a few disaccharide-repeating units and does not digest the monomeric Lipid II, formation of a PG strand is a pre-requisite in this assay. *S. aureus* PBP4 is a LMW PBP that does not have PGT activity, therefore, we coupled PBP4 with a monofunctional glycosyltransferase, SgtB to make nascent PG polymers.[34e] To evaluate if PBP4 has TP activity, we also added glycine oligopeptides (Gly_x: Gly_1, Gly_2, Gly_3 and Gly_5) to the reaction mixture to mimic the acceptor strands in cross-linking. The resulting muropeptide fragments were analyzed via LC/MS. As shown in Figure 2.4, SgtB alone gave only unmodified fragment **A**, confirming its involvement in nascent PG formation only (Figure 2.4b, trace i); SgtB and PBP4 produced two products in the absence of Gly_x: unmodified fragment **A**, and the hydrolysis product **B** (Figure 2.4b, trace ii). When Gly_x was added, *S. aureus* PBP4 preferentially incorporated the exogenous nucleophile to product $\mathbf{C}_x$ even though the concentration of water is many orders of magnitude higher (Figure 2.4b, trace iii-iv, and Figure App1.1). In addition, we also observed efficient D-amino acid incorporation when D-Ser, D-Phe or D-Tyr (1mM) was added. While mutating the PBP4 catalytic serine residue abolished all activity (Figure App1.2). The ability of PBP4 to discriminate against water and incorporate glycine oligopeptide and D-amino acids

[e] Previous studies have shown that SgtB polymerized Lys-Lipid II *in vitro* to long glycan polymers of approximately 50 disaccharide units.

shows that it acts as a TP *in vitro*, unlike most other LMW PBPs, which possess CP activity exclusively.[35]

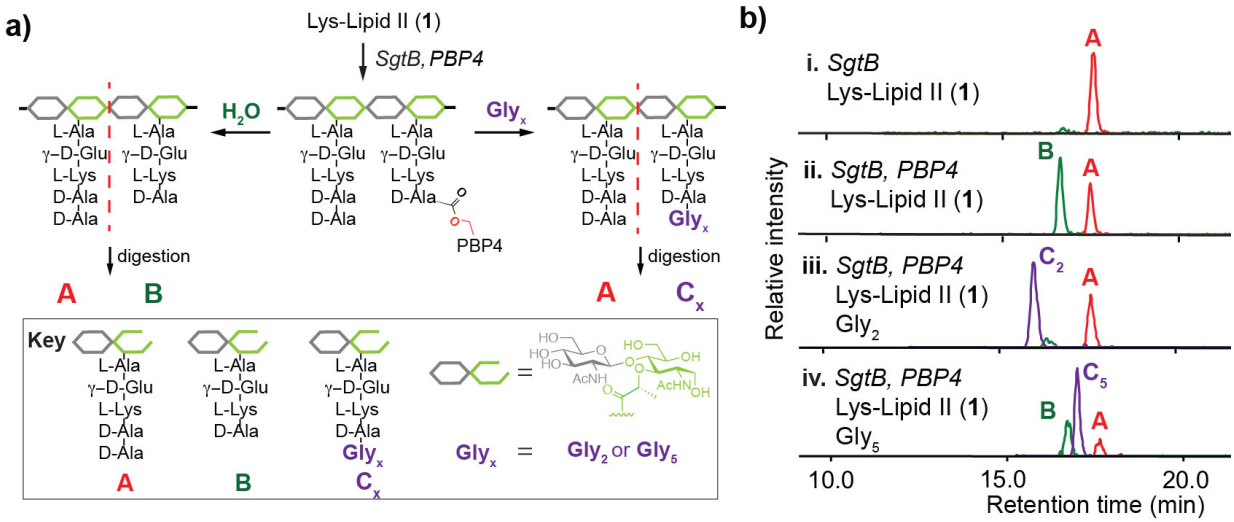

Figure 2.4. *S. aureus* PBP4 demonstrates TP activity *in vitro*. (a) Schematic of assay to monitor PBP4 activity. The PBP4-activated substrate adduct can be attacked by water or Gly_x. Three possible degradation products are yielded: **A** represents the unreacted muropeptide, **B** is the hydrolysis product, and **C** is a TP product with Gly_2 or Gly_5 incorporated. (b) LC/MS extracted ion chromatograms (EICs) of a control reaction without PBP4 (i), a reaction with PBP4 (ii), and reactions containing PBP4 and Gly_2 (iii) or D-Ser (iv). (M+2H)/2 ions: **A**, 485.2; **B**, 449.6; **C**(Gly_2), 506.7; **C**(Gly_5), 592.3. (See materials and methods for detailed protocols).

2.4 Assessment of *S. aureus* PBP4 *in vivo* activity

To assess if *S. aureus* PBP4 has TP activity in cells, we investigated the ability of wildtype *S. aureus* and the Δ*pbp4* mutant strains to incorporate a fluorescent D-Lys probe (FDL).[36] We observed that wildtype *S. aureus* cells were brightly labeled with FDL, with fluorescence concentrated at the crosswall where the bulk of PG synthesis takes place (Figure 2.5a).[37] The Δ*pbp4* strain showed only faint labeling, but complementation of *pbp4* gene restored efficient labeling. Monitoring fluorescence signals in *S. aureus* that were incubated with FDL for various periods of time (1min, 10min and 30min) indicates that FDL incorporation is efficient and the signal intensity increases over time (Figure 2.5). These results support that PBP4 acts as

a TP in cells and is primarily responsible for catalyzing D-amino acid exchange into PG in *S. aureus*.[f] Previous findings have shown that the level of PG cross-linking in *S. aureus* is drastically reduced in Δ*pbp4* mutant, implying its cellular function as a TP.[19] Our observation is consistent with this.

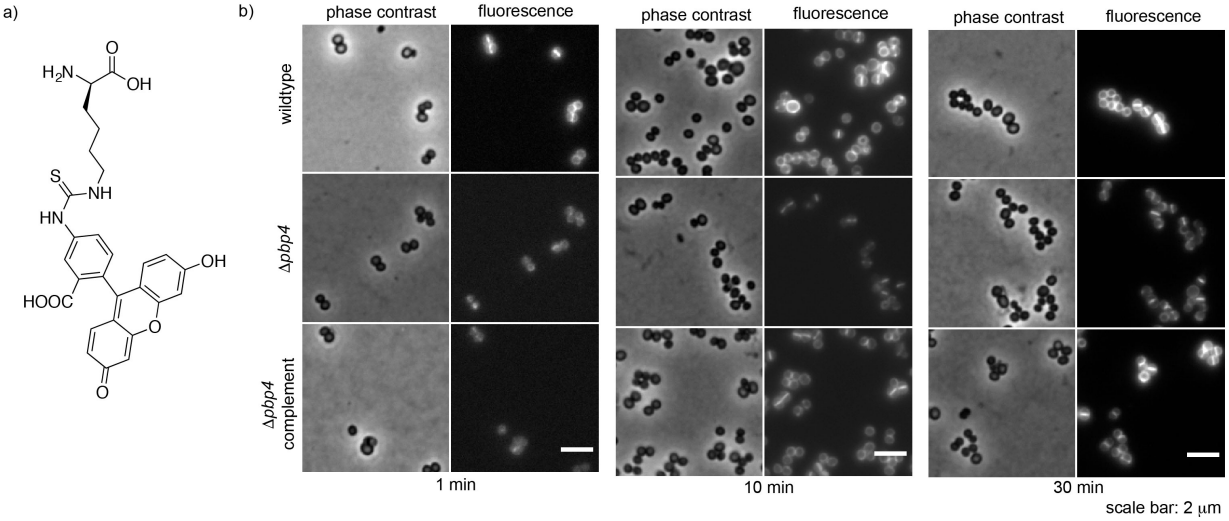

Figure 2.5. *S. aureus* PBP4 has TP activity *in vivo*. a) Chemical structure of fluorescent D-lysine probe (FDL). b) *S. aureus* wildtype strain incorporates FDL efficiently, but the Δ*pbp4* strain shows dramatic decrease in FDL incorporation. Complementation with *pbp4* gene restores the incorporation efficiency (Scale bar: 2 μM). Sample preparation is described in materials and methods.

Using fluorescent D-amino acid derivatives to study bacterial PG synthesis has been of great interest in recent years.[38,39] Different mechanisms are responsible for D-amino acid incorporation into PG in different bacteria. For instance, the L,D-transpeptidases in *E. coli* are primarily responsible for exogenous D-amino acid incorporation at the fourth position of the stem peptide;[40] whereas in *B. subtilis*, exogenous D-amino acids are incorporated by TPs into the

[f] The faint labeling associated with Δ*pbp4* strain indicates that other PBPs or pathways may play a role in FDL incorporation as well.

fifth position exclusively, but are rapidly hydrolyzed by CPs, and deletion of *dacA*[g] is necessary to retain fluorescent labels in PG.[36,40] In addition, *Chlamydia trachomatis*, a species whose PG is only recently appreciated, can incorporate fluorescent analogues of D-Ala-D-Ala dipeptide probes in PG via the intracellular ligase MurF.[39] In the case of *S. aureus*, the fluorescent D-amino acid was incorporated into the fifth position in the stem peptide in PG. Our work demonstrates that *S. aureus* PBP4 is the primary PBP responsible for D-amino acid incorporation in PG.[30] Two recent works also illustrate the importance of *S. aureus* PBP4 in fluorescent labeling of PG.[41,42]

2.5 Analysis of *S. aureus* PBP4 substrate specificity *in vitro*

For certain HMW PBPs, TP activity requires either ongoing PG synthesis or preformed uncross-linked PG polymers.[8,9,43] All previously studied LMW PBPs have less stringent substrate requirements that only require a tripeptide mimic for their CP activity.[44,45] In order to assess the substrate requirements of *S. aureus* PBP4, we modified the *in vitro* assay by adding enzymes sequentially. To determine if preformed PG is a PBP4 substrate, Lys-Lipid II was polymerized with SgtB, which was heat-inactivated prior to adding PBP4 and D-Tyr.[h] In a parallel set of experiments to test if Lipid II could be a substrate, Lys-Lipid II was first incubated with PBP4 and D-Tyr and then heat-inactivated prior to adding SgtB to polymerize the modified Lipid II. LC/MS analysis of the reaction products showed that D-Tyr was incorporated efficiently in both sets of reactions (Figure 2.6). Thus PBP4 uses both nascent PG and Lipid II monomers as substrates *in vitro*. This is the first demonstration of a TP capable of activating monomeric Lipid II. PBP4 has been suggested responsible for the formation of highly cross-linked PG in *S.*

[g] *B. subtilis* DacA encodes PBP5, a LMW PBP that acts as a carboxypeptidase.
[h] D-Tyr was chosen as an example here; other D-amino acids could be incorporated as well.

aureus.[19] We believe the promiscuity of *S. aureus* PBP4 in substrate recognition has important ramifications for its cellular functions.[46,47] The ability of *S. aureus* PBP4 to act on both glycan strands and Lipid II, independent of glycan polymerization, may allow it to add cross-links to partially cross-linked PG and to repair defects in cells. This role may be particularly important under stress conditions.[48]

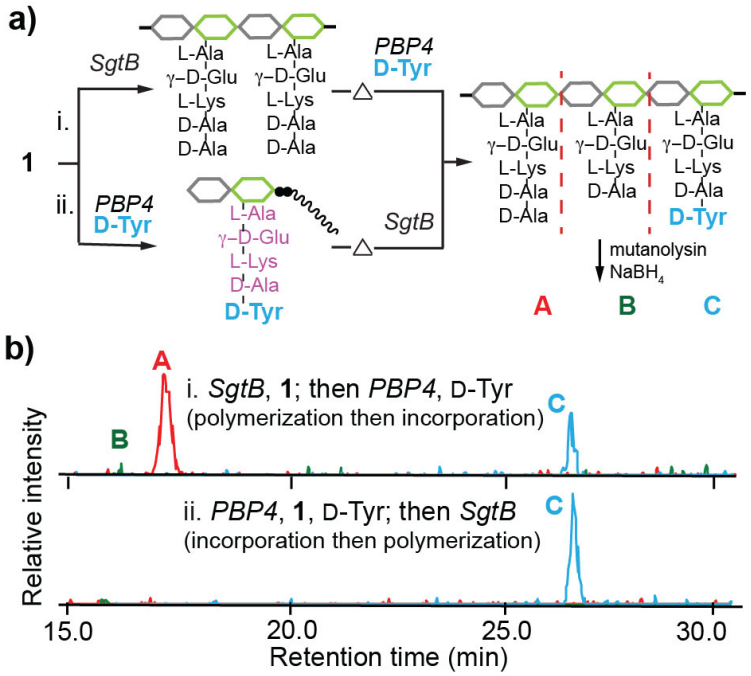

Figure 2.6. *S. aureus* PBP4 exhibits promiscuous TP activity *in vitro*. (a) Schematic for analyzing PBP4 substrate tolerance. (b) LC/MS EICs of PBP4 reactions with preformed PG (i) and Lipid II (ii) both show the D-Tyr-containing muropeptide peak C. (M+2H)/2 ions were extracted: **A**: 485.2, **B**: 449.6, **C**: 531.2. (See materials and methods for detailed protocols).

S. aureus PBP4 exchanges D-amino acid into Lys-Lipid II efficiently. We found that the presence of an increasing concentration of D-Tyr (at 0 μM, 20 μM, 400 μM, and 1mM) in the assay favors the formation of D-Tyr containing muropeptides. Approximately, a 10-fold excess of D-Tyr (400 μM) over Lys-Lipid II (40 μM) led to the complete exchange of the terminal D-Ala over one hour of reaction (Fig. App1.3). PBP4 can also efficiently incorporate other D-amino acids (such as D-Ser, D-Phe, D-Lys and so on) into Lys-Lipid II. To investigate if

different acceptors behave differently during incorporation, we set up a competition experiment where two nucleophiles, D-Ala(d_3) and Gly-Gly dipeptide were added to the reaction mixture in equal concentrations (at 1 mM each).[i] At various time points, the reaction was subjected to analysis on LC/MS (Figure 2.7). In this experiment, D-Ala(d_3), which resembles the terminal D-Ala but can be distinguished on MS, was used to probe the reverse reaction of activation; Gly-Gly dipeptide was used to represent the glycine branch in cross-linking. We observed that D-Ala(d_3) was quantitatively incorporated within the first 15 min of the reaction (Figure 2.7b, peak **C**), but was replaced by Gly-Gly as reaction proceeded (Figure 2.7b, peak **D**). As illustrated in Figure 2.7c, the incorporation of D-Ala(d_3) and Gly-Gly into Lipid II both involves the formation of a common acyl-enzyme intermediate. D-Ala(d_3) attacks the intermediate rapidly, forming a modified D-Ala-D-Ala(d_3) terminus in Lipid II, which can then be re-activated by PBP4 to yield the covalent intermediate again. Thus, the incorporation of D-Ala(d_3) is reversible. On the other hand, Gly-Gly incorporation occurs slower but is irreversible, since re-inactivation of the modified terminus with Gly-Gly by PBP4 was not observed. As a result, Lipid II was quantitatively incorporated with Gly-Gly after prolonged incubation. This observation is in accordance with the fact that PG cross-links are not hydrolyzed by PBPs.

Prompted by the promiscuous TP activity of *S. aureus* PBP4, we further examined the scope of synthetic Lipid I and Lipid II analogues as substrates. We found that PBP4 can act on canonical *E. coli* Lipid II, which contains an *m*-Dap in place of L-Lys in the stem peptide (Figure App.1.3). In addition, Lipid I analogues could be readily modified by PBP4 via D-amino acid exchange and then converted to the corresponding Lipid II analogues in the chemoenzymatic MurG reaction (Figure 2.8). Using biotinylated D-Lys (BDL), we readily obtained BDL-Lipid I

[i] The reaction contains 2 μM of *S. aureus* PBP4 and 40 μM of Lys-Lipid II.

and Lipid II. A number of Lipid I and II analogues with unnatural D-amino acid in the terminal stem peptide position, such as D-propargylglycine and D-7-azatryptophan were prepared similarly (Figure App1.4 and 1.5). However, incorporation of D-amino acid derivatives with bulky side chains that are close to the alpha carbon (Cα), such as D-Ala-7-amino-4-mehtylcoumarin (AMC), was impeded, whereas derivatives with the bulkiness further away from Cα (such as BDL) was well tolerated by *S. aureus* PBP4.

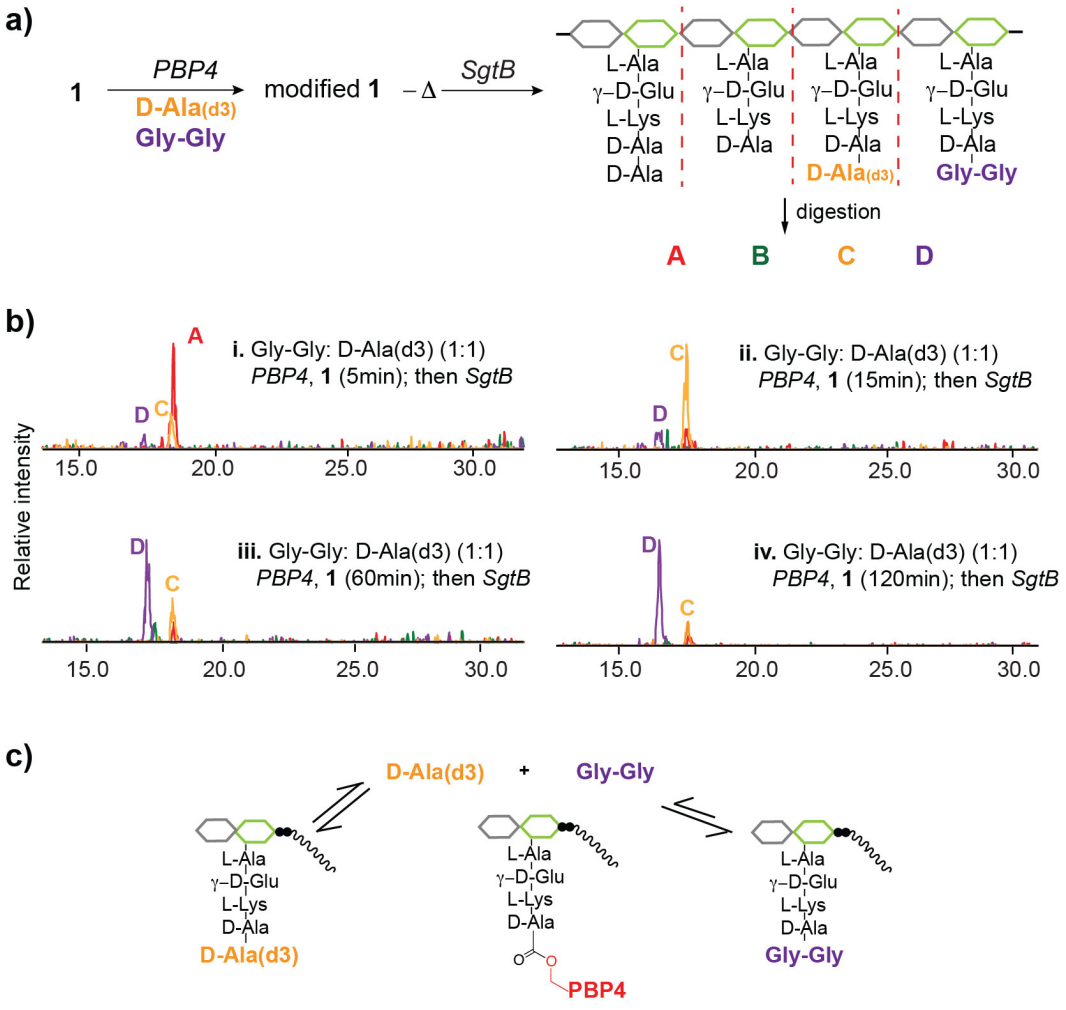

Figure 2.7. LC/MS analysis of *S. aureus* PBP4-mediated D-amino acid exchange in Lipid II. (a-b) Schematic and EICs of PBP4 reactions with Lys-Lipid II in the presence of an equal amount of D-Ala(d_3) and Gly-Gly as acceptors. D-Ala(d_3) incorporated muropeptide (**C**) was observed wihin 15 min of incubation, but D-Ala(d_3) was replaced by Gly-Gly (**D**) after prolonged incubation (3 h). (M+2H)/2 ions were extracted: **A**: 485.2, **B**: 449.6, **C**: 486.7, **D**: 506.7. (c) Schematic showing differences between

(Continued) D-Ala(d_3) and Gly-Gly incorporations into Lipid II based on experimental data in (b). D-Ala(d_3) incorporation is faster but reversible; whereas Gly-Gly incorporation is slower but stable. (See materials and methods for detailed protocols).

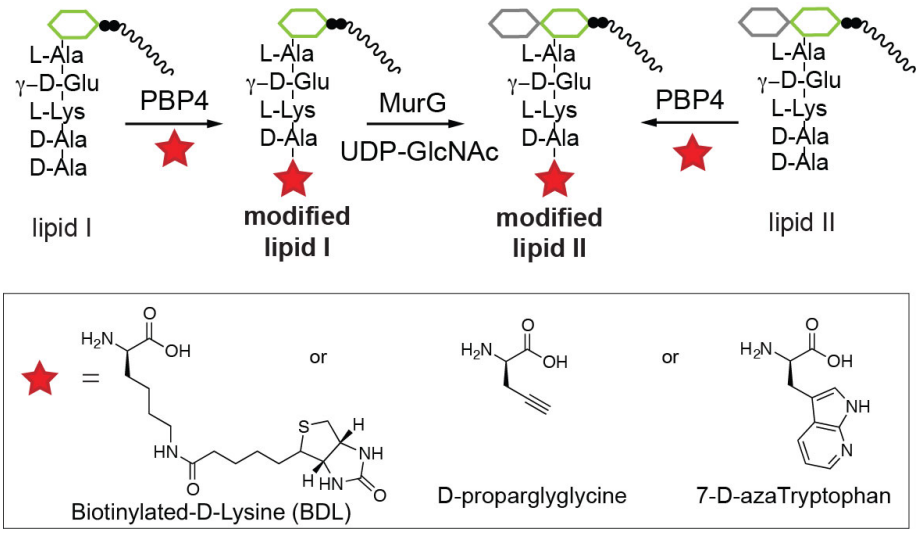

Figure 2.8. Schematic showing the versatility of PBP4 TP activity. PBP4 can incorporate a variety of functionalized D-amino acids into either Lipid I or Lipid II *in vitro*.

2.6 Detection of the cellular levels of Lipid II in bacteria

The unexpected TP activity of PBP4 to label Lipid I and Lipid II may facilitate development of assays to study PG processes. We wondered if PBP4's ability to biotinylate Lipid II precursors can be used to detect the cellular Lipid II in bacteria, which has been a longstanding challenge, since previous assays were laborious and involved the use of radioactivity.[21-23] As shown in Figure 2.9, we extracted cellular lipids from a small *S. aureus* culture (2mL) at mid-log phase (O.D.= 0.4-0.5) using chloroform and methanol solvents in a Bligh-Dyer extraction similar to the report by Guan *et al.*[49] To selectively biotinylate Lipid II in the lipid extract, we incubated *S. aureus* PBP4 and BDL with the extract (which was resuspended in a DMSO solution) for one hour. After reaction, the mixture was quenched and subjected to gel electrophoresis, followed by blotting with a streptavidin-HRP conjugate.

Remarkably, biotinylated Lipid II species were readily detected after a brief exposure (~2 seconds). We note that both cellular Lipid I and Lipid II may be labeled by PBP4, but since bacteria has an excess of Lipid II than Lipid I, we assumed that the signal mainly came from labeling of cellular Lipid II. MS analysis described in Chapter Three confirms that only Lipid II was extracted from *S. aureus* in our studies.

To demonstrate the utility of our assay, we assessed the cellular levels of Lipid II in *S. aureus* when treated with different antibiotics. As shown in Figure 2.9, moenomycin (MmA) and vancomycin (Vanco) treatments increased the Lipid II pool levels, presumably because Lipid II is no longer consumed by PGTs and therefore accumulates on the external surface of the cell membrane. Our observation was consistent with previously studies on the accumulation of radiolabeled PG precursors in moenomycin (MmA)- and vancomycin (Vanco)- treated bacteria.[23] In contrast, fosfomycin (Fos), targocil (Tar) and bacitracin (Bac) treatment all reduced the pool levels. Fosfomycin inhibits MurA activity, which is an early step in Lipid II synthesis,[50] thus is expected to decrease the cellular Lipid II level. Targocil (Tar) targets TarGH, the wall teichoic acid (WTA) flippase, and inhibits the translocation of WTA precursors across the membrane,[51-53] leading to an accumulation of WTA intermediates in the inner leaflet of the membrane in cells. Since both Lipid II and WTA use undecaprenyl phosphate (Und-P) as the lipid carrier, accumulation of WTA precursors would limit the availability of Und-P for Lipid II synthesis.[54] Bacitracin, which specifically binds to the undecaprenyl pyrophosphate (Und-PP) in the outer leaflet of the membrane, prevents dephosphorylation and hence recycling of the lipid carrier to the inner leaflet for Lipid II biosynthesis,[55] thus depleting the cellular Lipid II levels in *S. aureus*. However, a minimal change in Lipid II level was observed in *S. aureus* treated with kanamycin (kan), a non PG-targeting antibiotic (Figure 2.9). Therefore, using PBP4-mediated

biotinylation, we developed a simple and sensitive assay to study the cellular Lipid II levels in *S. aureus*. This assay should facilitate studies on the mechanism of action of potential PG-targeting antibiotics.

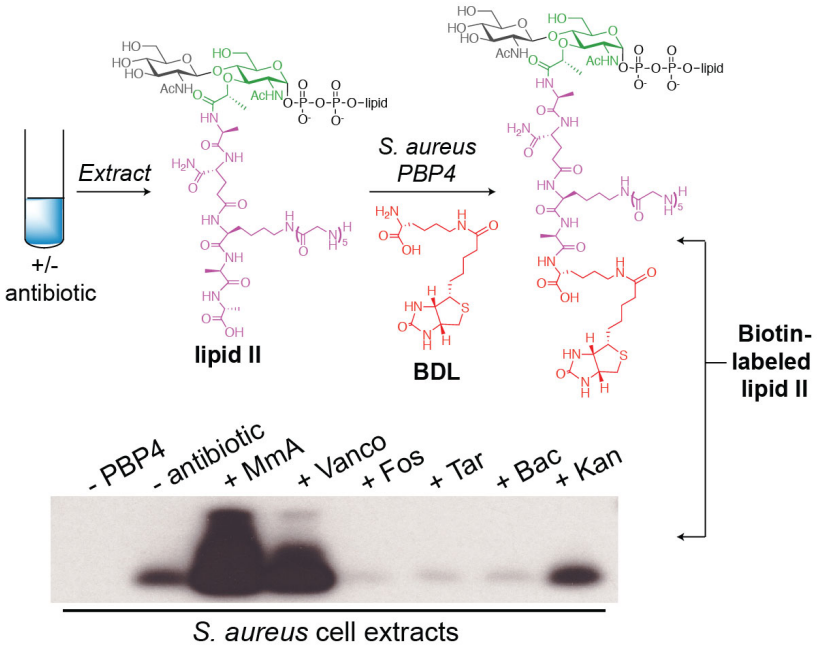

Figure 2.9. *S. aureus* PBP4 enables a simple assay to detect cellular Lipid II in bacteria. Antibiotic treatments cause changes in the pool levels of Lipid II in *S. aureus*, which are consistent with their mechanisms of action.

2.7 Elucidation of the cellular mechanism of action for lysobactin

In collaboration with a postdoctoral researcher in the Walker lab, Wonsik Lee, we applied the Lipid II detection assay described above to characterize the cellular mechanism of lysobactin. Lysobactin is a macrocyclic depsipeptide natural product that is produced by a species of Lysobacter (ATCC 53042).[56] It displays excellent antibacterial activity against a number of drug-resistance bacteria, including MRSA, vancomycin-resistant enterococci (VRE), as well as *Mycobacterium tuberculosis* (TB) with its MICs being significantly lower than that of vancomycin (2- to 50- fold lower depending on the strains). While several total syntheses of lysobactin and its biosynthetic pathway have been reported,[57-61] its mechanism of action of is still

poorly investigated. Lysobactin was originally identified as a binder to the crude preparations of *S. aureus* cell wall; however, its mechanism appeared distinct from vancomycin since lysobactin did not bind to the lysozyme-solubilized cell wall (whereas vancomycin did).[62] In macromolecular synthesis assays, lysobactin inhibited [^{14}C]-*m*-Dap incorporation into PG but had no effects on either [^{14}C]-uridine or [^{14}C]-thymidine incorporation in RNA or DNA synthesis. However, the exact mechanism of action of lysobactin has remained unclear when this project was initiated in the Walker and Kahne labs.

In the *in vitro* activity assays of several enzymes involved in PG synthesis (i.e. MurG, SgtB, TagB), lysobactin demonstrated enzymatic inhibition with a characteristic substrate-binding curve.[63j] Unlike previous known substrate-binding inhibitors such as ramoplanin[64] and teixobactin[65] that have a stoichiometry of 2:1 (drug: cell wall precursors), lysobactin gives a distinctive 1:1 stoichiometry in the *in vitro* assays. We learned that lysobactin binds to the sugar-pyrophosphate moiety in several cell wall precursors *in vitro*, including Lipid I, Lipid II and WTA precursors; however, identifying the cellular targets of lysobactin responsible for its antibacterial activity *in vivo* requires cellular assays. The *S. aureus* PBP4-enabled detection of cellular Lipid II in bacteria (see Chapter 2.6) allows us to elucidate the cellular effects of lysobactin. We treated *S. aureus* culture (2 mL) with lysobactin at 1.5 μg/mL or 3 μg/mL (i.e. 2x or 4x of its MIC) for 10 min, extracted the cellular lipids and labeled with BDL using *S. aureus* PBP4. As shown in Figure 2.10, a dramatic accumulation of Lipid II was observed upon lysobactin treatment. The higher bands were products of PBP4 cross-linking of Lipid II during BDL labeling, which disappeared upon treatment with lysostaphin, an endopeptidase that

[j] In such inhibition curves, the enzymatic reaction rate is negligible at low substrate concentration because of no free substrate but jumps soon as substrate becomes available. Wonsik Lee performed the *in vitro* enzymatic assays. Wonsik Lee and I performed the *in vivo* Lipid II accumulation assays.

specifically cleaves cross-linked pentaglycine bridges.[63] Therefore, we conclude that lysobactin preferably binds to Lipid II in cells, contributing to its cellular mechanism of action. Although lysobactin has the ability to bind to WTA precursors *in vitro*, WTA substrates are not the cellular targets of lysobactin. Given that only one in nine MurNAc residue in PG is modified with WTA, the precursor flux is substantially higher through the PG pathway in bacteria.[66] Lipid II is the cellular target of lysobactin.

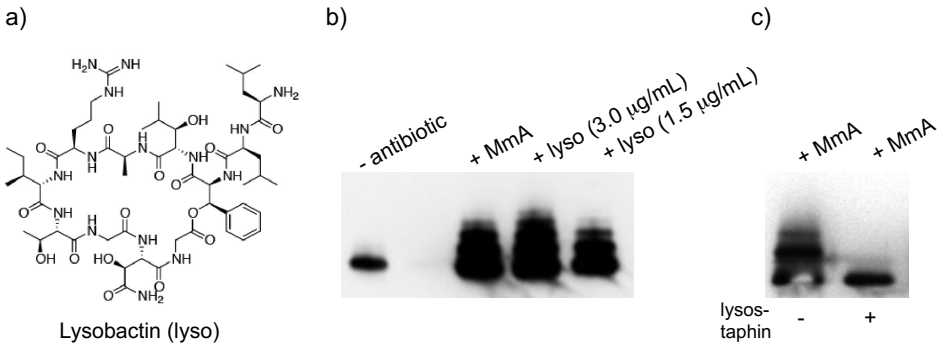

Figure 2.10. Lysobactin treatment accumulates Lipid II in *S. aureus*. a) Structure of lysobactin (lyso). b) Western blot of BDL-labeled Lipid II in *S. aureus* showed the accumulation of cellular Lipid II in lysobactin-treated *S. aureus*. The multiple bands were due to PBP4 crosslinking of concentrated Lipid II. c) Lysostaphin treatment of multiple bands resulted in collapsing of the bands.

2.8 Conclusion

In conclusion, through studies of *S. aureus* PBP4 activity *in vitro* using Lys-Lipid II and various oligoglycines or D-amino acids as acceptors, we have established that PBP4 is a transpeptidase (TP) with unique activity to act on monomeric Lipid II substrate. Since its TP activity is remarkably different from other previously characterized PBPs, it is possible that other LMW PBPs possess similar TP activity as *S. aureus* PBP4. While most LMW exhibit solely carboxypeptidase or endopeptidase activities, *Streptomyces* LMW PBPs (R61 and R39) have been also characterized as TPs *in vitro* with Ac_2-L-Lys-D-Ala-D-Ala tripeptide substrate.[35] In *Streptomyces*, the PG cross-link is made of a Gly attached to the amino side chain of LL-DAP at

the third position of the stem peptide. *Stretomyces* R61 and R39 were shown to incorporate a dipeptide acceptor (Gly-L-X_{aa}) into the tripeptide substrate, yielding a tetrapeptide product. In addition, R61 displayed weak reversible TP activity (i.e. hydrolysis) with the tetrapeptide (Ac_2-L-Lys-D-Ala-Gly-L-X_{aa}).[67] In our case, *S. aureus* PBP4 did not hydrolyze the transpeptidation product.[k] In addition, for *S. aureus* PBP4 activities, the presence of a free carboxylate at Cα in the acceptor is not an absolute prerequisite, since we observed that the methyl ester derivatives of oligoglycines could be incorporated by PBP4; however, the effects of the carboxylate modification may depend on the acceptor structure, since the carboxamide derivatives were discriminated by PBP4 *in vitro*. The acceptor substrate specificity of *S. aureus* PBP4 in our studies is similar to that of R61 reported previously.[67]

Exploiting the unexpected transpeptidase activity of *S. aureus* PBP4, we developed a simple and sensitive assay to detect the cellular levels of Lipid II in bacteria, addressing a longstanding challenge. We observed that treatment of various cell wall-targeting antibiotics led to changes in the cellular pools of Lipid II in *S. aureus*, consistent with their known mechanisms of action. In particular, antibiotics such as moenomycin and vancomycin that inhibit late steps in PG synthesis resulted in an accumulation of Lipid II, whereas antibiotics that either target an earlier step in the pathway (e.g. fosfomycin) or interfere with the carrier lipid (Und-P) recycling (e.g. bacitracin and targocil) deplete Lipid II. Hence, we now have a method to determine the cellular mechanisms of potential antibiotics based on their effects on Lipid II levels. As a demonstration, we have established that lysobactin, a macrocyclic depsipeptide natural product inhibitor, strongly accumulates Lipid II in *S. aureus*. This observation is in line with the proposed mechanism of lysobactin as a Lipid II binder in cells. We are currently using this assay

[k] Except when D-Ala(d_3) and Gly_2 acceptors were both present in the reaction, D-Ala(d_3) was incorporated and re-activated, followed by Gly_2 incorporation (Figure 2.7).

to profile the effects of potential PGT inhibitors on Lipid II levels in *S. aureus*. In closing, we note that the Lipid II detection assay using *S. aureus* PBP4 can also be applied to other organisms, including *E. coli*, *E. faecalis*, *B. subtilis* and *Acinetobacter baumannii*, opening up the possibility to study PG-related processes in various organisms.

2.9 Materials and Methods

2.9.1 Materials

Lys-Lipid I, Lys-Lipid II, and *m*-Dap-Lipid II were prepared as previously described.[13,32,43] N-terminus truncated *S. aureus* SgtB were purified as reported previously.[68,69] Fluorescein D-Lysine (FDL) was prepared as described.[36] *S. aureus* PBP4 deletion strain *(*Δ*pbp4*) and the complementation strain (Δ*pbp4* complemented with *pbp4* gene) were obtained from the Cheung Lab.[70] Primers were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from New England Biolabs. Vectors and expression hosts were obtained from Novagen. Non-stick conical vials and pipette tips used for enzymatic reactions were purchased from VWR. H-Gly-Gly-OH and H-Gly-Gly-Gly-OH were purchased from TCI. H-Gly-Gly-Gly-Gly-Gly-OH was purchased from Bachem. D-7-Azatryptophan was purchased from Santa Cruz Biotechnology Inc. Fmoc-D-Lys(biotinyl)-OH was purchased from VWR. D-Propargylglycine (DPG) was purchased from Peptech Corporation. D-Alanine-3,3,3-d_3 (D-Ala-d_3) was purchased from CDN Isotopes. Bocillin-FL penicillin, Na salt (Bo-FL) was purchased from Invitrogen. Moenomycin A was isolated from flavmoycin feedstock as previously reported.[71] All other chemicals were purchased from Sigma Aldrich unless otherwise noted.

2.9.2 General Methods

Low-resolution mass spectra chromatograms were obtained on an Agilent Technologies 1100 series LC-MSD instrument using electrospray ionization (ESI). High-resolution mass spectra data was obtained on an Agilent 6520 LC-MS Q-TOF mass spectrometer instrument using ESI. Microscopic images were acquired with a Hamamatsu digital camera model ORCA-ER connected to a Nikon Eclipse TE2000-U microscope with X-cite 120 illumination system. A typhoon phosphorimager (GE Healthcare) was used to capture fluorescent SDS-PAGE gels. ImageJ was used to analyze microscopy images and western blots.

2.9.3 Plasmid construction

The *pbp4*[Y21-Q383] gene encoding truncated *S. aureus* PBP4[Y21-Q383], lacking the predicted transmembrane segments was PCR purified from *S. aureus* Newman strain using the following primer pair: 5'-CCTAGCTAGCTATGCACAAGCTACTAAC-3' and 5'-GCAGGGATCCTTACTGATGAACTTCTAC-3'. After digestion with NheI and BamHI (the restriction sites are underlined), the PCR fragment was ligated into pET28a(+) to produce pPBP4[Y21-Q383] as a N-terminal His_6 fusion. The inserted *pbp4*[Y21-Q383] gene was confirmed by sequencing (Genewiz or Beckman Sequencing Facility). *E. coli* novablue strain was used for cloning.

The catalytic serine residue (S75) of the transpeptidase domain of *S. aureus* PBP4 has been identified by sequence alignment with other PBPs.[72] The S75A mutation was made in the parent plasmid pPBP4[Y21-Q383] using techniques described in the QuickChange site-directed mutagenesis kit (Stratagene) to make the mutant plasmid pPBP4[Y21-Q383] S75A with the forward primer 5'-CTAAGTGGAATCCAGCGGCAATGACTAAATTAATGAC-3' and the

reverse primer 5'-GTCATTAATTTAGTCATTGCCGCTGGATTCCACTTAG-3' (mutagenized codon is underlined).

2.9.4 Overexpression and purification of *S. aureus* PBP4 and mutant

Plasmid pPBP4[Y21-Q383] encoding for soluble *S. aurues* PBP4 and plasmid pPBP4[Y21-Q383] S75A encoding for *S. aureus* PBP4 TP* were collected from the cloning strains, and were transformed into *E. coli* BL21(DE3) cultures respectively for overexpression and purification. *E. coli* BL21 (DE3) cultures (LB medium supplemented with 50 μg/ mL kanamycin) were used to inoculate 1 L of LB medium (1:100) supplemented with 50 μg/mL kanamycin at 37 °C and grown to OD_{600} = 0.4-0.5 with shaking. Cells were cooled to 17 °C before induction with 0.5 mM IPTG for 17 h with shaking. Cells were harvested by centrifugation (5250 x g, 20 min, 4 °C) and pellets were resuspended on ice with 30 mL of Buffer A (20 mM Tris (pH = 7.5), 400 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by three passages through a cell disrupter (3 x 10,000 psi, 4 °C). The cell lysate was then pelleted by ultracentrifugation (90,000 x g, 30 min, 4 °C). The resulting supernatant containing PBP4 protein was added to 1.5 mL washed Ni-NTA resin (Qiagen) and rocked at 4 °C for 40 min. After loading the column, the resin was washed with wash buffer (20 mL Tris (pH = 7.5), 40 mM NaCl, 0.1 % Triton-X-100, 40 mM imidazole). The protein was eluted with 200 mM imidazole in 10 mL of elution buffer (20 mM Tris (pH = 7.4), 500 mM NaCl, 0.1% reduced Triton-X-100, 200 mM imidazole). The eluent containing His_6-PBP4 was concentrated to < 1 mL using 30 kD MWCO Amicon Ultra Centrifuge Filter Devices (Millipore). The yield of purified protein was found to be ~ 8 mg/mL by DC protein assay (Biorad). Proteins were stored at -80 °C.

2.9.5 Bocillin-FL binding assay

Purified *S. aureus* PBP4 protein (250 nM) was incubated with varying concentrations of penicillin G (penG) (1000, 100, 0 U/mL) in a buffer containing 20 mM potassium phosphate (pH = 7.5) and 140 mM NaCl (to reach a volume of 9 μL). The mixture was incubated at 37 °C for 1 h with shaking. Bocillin-FL (1 μL of 100 μM stock) was added to the mixture (total reaction volume is 10 μL),[73] which was then incubated for 30 min at 37 °C with shaking. The reaction was quenched by adding 2x SDS loading dye, and heated for 20 min at 95 °C. The samples were loaded onto a SDS-PAGE gel, which was then scanned using Typhoon (fluorescence scan, excitation 488 nm, emission 526 nm). The scanned image was then analyzed using ImageJ (Figure 2.3).

2.9.6 LC/MS protocol for muropeptide analysis

The procedure was modified from a recent report by Lebar *et al.*[12] Lys-Lipid II (40 μM) was incubated with SgtB (1 μM) and PBP4 (2 μM) in reaction buffer (12.5 mM HEPES (pH = 7.5), 2 mM $MnCl_2$, 20% DMSO, and 250 μM Tween-80) in a total of 10 μL reaction volume for 1 h at 25 °C in the absence or presence of glycine oligopeptides or various D-amino acids (all solutions were used at 1 mM; except for Gly_3 and Gly_5 solutions that were acidified with 0.01 N HCl to facilitate solubility, and were used at a final concentration of 5 mM). The reaction was quenched at 95 °C for 5 min, and then treated with mutanolysin (from *Streptomyces globisporus*, Sigma, 1 U) for 1.5 h at 37 °C followed by another 1 U aliquot for 1.5 h. The resulting disaccharides were reduced with sodium borohydride (10 mg/mL, 30 min) to resolve the MurNAc anomers. Phosphoric acid (20%, 1.2 μL) was then added to adjust the pH to ~ 4. The reaction mixture was the lyophilized, redissolved in 12 μL H_2O and subjected to LC/MS

analysis, conducted with ESI-MS operating in positive mode. The instrument was equipped with a Waters Symmetry Shield RP18 column (5 μM, 3.9 x 150 mm) with a matching column guard. The fragments were separated using the following method: 0.5 mL/min H_2O (0.1% formic acid) for 5 min followed by a gradient of 0% acetonitrile (ACN) (0.1% formic acid)/H_2O (0.1% formic acid) to 20% ACN (0.1% formic acid)/H_2O (0.1% formic acid) over 40 min. Molecular ions corresponding to expected disaccharide fragments were extracted from chromatograms (Figure 2.4 and Figure App1.1).

To analyze the ability of PBP4 to use polymeric PG and monomeric Lipid II as substrates, the above procedure was modified slightly. To analyze PBP4 activity on preformed PG polymer, Lys-Lipid II (40 μM) was incubated with SgtB (1 μM) for 1 h at 25 °C, prior to inactivation of SgtB at 95 °C for 5 min. PBP4 (2 μM) and D-Tyr (1 mM) were added to the reaction mixture which was incubated for another h at 25 °C before heat quenching. The digestion procedure was identical to that discussed above. To analyze PBP4 activity on Lipid II monomer, Lys-Lipid II (40 μM) was incubated with PBP4 (2 μM) and D-Tyr (1 mM) for 1 h at 25 °C, prior to inactivation of PBP4 at 95 °C for 5 min. SgtB (1 μM) was added to the reaction mixture which was incubated for another h at 25 °C before heat quenching. The digestion procedure was identical to above. Molecular ions corresponding to the expected disaccharide fragments were extracted from chromatograms (Figure 2.6). To analyze PBP4 activity to exchange unnatural D-amino acids into Lipid II monomers, the respective D-amino acid acceptors (1 mM) were used (Figure 2.7 and Figure App1.2).

To analyze PBP4 activity on D-amino acid incorporation into Lipid II at varying concentrations of the D-amino acid, D-Tyr (0 μM, 20 μM, 400 μM and 1 mM) was used in the reaction as discussed above (Figure App1.3).

2.9.7 Microscopy imaging analysis

S. aureus strains (MW2 wildtype, Δ*pbp4*, and Δ*pbp4* complemented with *pbp4* gene)[74] were grown for 4 h in TSB at 37 °C to stationary phase. The cultures were then diluted to reach OD_{600} = 0.1 and allowed to grow for an additional 30 min to reach mid-log phase. To 0.5 mL of *S. aureus* culture, 1 μL of FDL probe (2 mM in DMSO) was added (giving a final concentration of 4 μM). The cultures were incubated briefly for 10 min at 37 °C with shaking, and fixed with ice cold 70% ethanol immediately. The fixed cells were then incubated on ice for 15 min, before washing with PBS buffer for three times. The washed cell pellet was resuspended in 30 μL PBS, and 2 μL liquid was mounted on PBS pads containing 2% agarose. The samples were imaged using BODIPY-GFP channel. Images were adjusted to the same intensity scale to allow comparison on ImageJ (Figure 2.5).

2.9.8 Preparation of BDL probe

To Fmoc-D-Lys(biotinyl)-OH (0.016 mmol, 10 mg) in a glass vial, 2 mL of 20% piperidine/DMF and 0.3 mL toluene were added. The mixture was stirred to dissolve for 30 min at room temperature, and then concentrated to remove solvent *in vacuo*. The crude concentrated mixture was diluted in H_2O leaving a white precipitate (deprotected Fmoc byproduct), which was removed via filtration. The filtrate was concentrated, and the product was analyzed by MS direct injection in positive mode. Pure BDL probe was prepared as a stock solution of 10 mM in H_2O.

2.9.9 Incorporation of functionalized D-amino acids into Lipid I and Lipid II

An aliquot of C20-Lys-Lipid I analogue (50 nmol) in DMSO (20 μL) was briefly sonicated and then diluted with 20 μL of 10x reaction buffer (125 mM HEPES pH = 7.5, 20 mM

$MnCl_2$, and 2.5 mM Tween-80). Biotinylated D-Lysine (BDL) (5 mM, final concentration) and PBP4 (20 μM, final concentration) were then added to the reaction mixture to reach a final volume of 200 μL. The reaction was incubated for 3 h at 25 °C, and then kept on ice before purification. The reaction mixture was loaded onto a pre-equilibrated Strata C18-E SPE column (Phenomenex), then eluted with ACN/25 mM NH_4HCO_3 using a step gradient of 0% to 100% ACN (500 μL each, 10% increment). The collected fractions were analyzed by MS direct injection in negative mode. The modified-Lipid I analogue was eluted in 30%-40% ACN fractions. HRMS data was obtained for the fractions contain pure BDL-incorporated Lipid I analogue (Figure App1.3-1.4). D-propargylglycine (DPG)-modified Lipid I analogue was obtained using DPG in place of BDL following the same protocol (Figure App1.3-1.4).

The fractions that contained pure BDL-incorporated Lipid I analogue were combined and concentrated by speed-vac. Lipid I to Lipid II conversion was carried out as previously described.[75,76] Briefly, the dried Lipid I analogue (50 nmol) was re-dissolved in MeOH (24 μL), and then added 23 μL of 7 x MurG buffer (350 mM HEPES pH 7.9, 35 mM $MgCl_2$), 5 μL UDP-GlcNAc (10 mM), and 96 μL H_2O. The mixture was vortexed before adding 8 μL alkaline phosphatase (20U/ μL, Roche) and 4 μL purified MurG (20 mg/mL) to reach a total final volume of 160 μL. After incubation at 25 °C for 1 h, another aliquot of 4 μL MurG (20 mg/mL) was added to the reaction to incubate for another 1 h. Cold H_2O (160 μL) was added to quench the reaction. The quenched mixture was loaded onto a pre-equilibrated BAKERBOND SPE C18-extraction column (J. T. Bakers), and washed with H_2O/ 0.1% NH_4OH (500 μL x 7), and then eluted with MeOH/ 0.1% NH_4OH (500 μL x 7). The fractions were analyzed by MS direct injection in negative mode. The modified-Lipid II analogue was eluted in the initial MeOH/ 0.1% NH_4OH fraction (500 μL). HRMS data was also obtained for the pure BDL-modified Lipid

II analogue. DPG-modified Lipid II analogue was converted from DPG-Lipid I following the same protocol (Figure App1.3-1.4).

2.9.10 Western blot detection of BDL-Lipid II *in vitro*

To a non-stick reaction vial, 3 μL H_2O and 1 μL of 10x reaction buffer (125 mM HEPES pH = 7.5, 20 mM $MnCl_2$, and 2.5 mM Tween-80) was added with 3 μL of BDL probe (10 mM in H_2O). The mixture was vortexed before adding 1 μL of Lys-Lipid II (200 μM in DMSO), 1 μL of DMSO, and 1 μL of PBP4 (40 μM) to reach a total volume of 10 μL. The reaction was incubated at 25 °C for 1 h. To quench the reaction, 10 μL of 2x SDS loading buffer was added and the samples were heated at 95 °C for 5 min. 3 μL of the final mixture was loaded onto a 15% SDS polyacrylamide gel. The products were transferred to Immun-Blot PVDF membrane (BioRad). BDL-Lipid II was detected by blotting with streptavidin-HRP (1:10000 dilution, Pierce), and visualized using ECL Prime Western Blotting Detection Reagent (GE Ambersham) and Biomax Light Film (Kodak). To confirm the presence of PBP4, the membrane was also blotted with anti-His_5-HRP (1:10000 dilution, Qiagen).

2.9.11 Extraction of cellular lipid-linked PG precursors and Western blot detection

The extraction of cellular lipids has been modified from a previously published protocol.[49] An overnight culture of *S. aureus* RN4220 was diluted to an OD_{600} = 0.1, and allowed to grow to mid-exponential phase at 37 °C. The culture was divided into 2 mL aliquots subsequently which were treated with antibiotics (concentrations of antibiotics were about 2x of their respective MICs against *S. aureus* RN4220). Following 10 min of growth at 37 °C, cells were harvested by centrifugation for 10 min at 5,000 x g. The amount of cell pellet collected was

normalized by OD_{600}, and resuspended in 500 µL PBS (pH 7.4). The resuspended pellet was transferred to a glass tube alongside 500 µL $CHCl_3$ and 1 mL MeOH. The mixture was vortexed repetitively for 10 min at 25 °C, and cell debris was removed with centrifugation for 10 min at 4,000 x g. The supernatant was collected and transferred to a new glass tube with 500 µL $CHCl_3$ and 500 µL PBS. The mixture was vortexed for 10 min, and centrifuged for 1 min at 4,000 x g to achieve phase separation. 1 mL of the organic layer was collected and concentrated *in vacuo*. The dried fractions were resuspended in 12 µL DMSO. Each fraction (2 µL) was used (in place of Lipid II and DMSO) in a BDL labeling reaction as described earlier. Reactions were further analyzed on Western blot with HRP-streptavidin conjugate (Figure 2.9 and 2.10).

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Chapter Three: Preparation of native *Staphylococcus aureus* Lipid II

Data contributed by collaborators:

Jeep Veerasak Srisuknimit and I performed studies on large-scale extraction of native Lipid II from bacteria.

3.1 Introduction

The inaccessibility to native Lipid II substrate has hampered enzymatic studies on the transpeptidase (TP) activities of bacterial PBPs. The structure of native Lipid II varies in bacteria; therefore, it is impossible to prepare a 'universal' Lipid II substrate and it is onerous to prepare new Lipid II variants by either chemical synthesis or enzymatic reconstitution. In this chapter, I first describe my attempts to synthesize *Staphylococcus aureus* (*S. aureus*) Lipid II, and then describe a general strategy to accumulate and isolate Lipid II from bacteria. Using the second approach, we can obtain useful quantities of native Lipid II from different bacteria within a day of work, greatly easing the challenges with Lipid II preparation. This facile strategy for Lipid II preparation was developed based on our findings described in Chapter Two, and in turn enables biochemical characterization of *S. aureus* PBP2 that is described in Chapter Four.

S. aureus Lipid II has one of the most complex structures among all bacteria Lipid II, bearing a pentaglycine branch connected to the L-Lys side chain as well as a carboxamide at the D-*iso*Gln on the stem peptide (Figure 1.4).[1] Although Strominger and co-workers have correctly predicted the complete structure of *S. aureus* Lipid II in the 1960s, obtaining useful amount of native Lipid II has not been possible for several decades,[2] posing a major challenge for biochemical characterizations of *S. aureus* PBPs. There are only two previous reports on preparations of the pentaglycine-containing Lipid II. Schneider *el al*. (2004) reported the *in vitro* assembly of the complete pentaglycine branch on Lipid II using purified FemXAB proteins and glycyl-tRNA donors.[3] More recently, Patin *et al*. (2012) reported chemical installation of the pentaglycine onto the Park nucleotide using activated glycines: Fmoc-Gly-OSu and Fmoc-Gly_2-OSu (twice) successively, after which the modified nucleotide was converted to Lipid I and Lipid II using coupled MraY and MurG reactions *in vitro*.[4] While both works provided valuable

information on the formation of the pentaglycine branch, the carboxamide on the D-*iso*Gln at the second position of the stem pentapeptide was omitted. In 2012, Munch *et al.* identified and reconstituted GatD/MurT enzymes, which are responsible for amidation on D-*iso*Gln of Lipid II in *S. aureus*. However, the native Lipid II substrate was obtained only in small amounts for analytical studies.[5] The proper amidation on D-*iso*Gln in *Streptococcus pneumoniae* (*S. pneumoniae*) Lipid II has been shown to be necessary for *S. pneumoniae* PBP transpeptidase (TP) activity.[6,7] Conceivably, both pentaglycine and carboxamide on *S. aureus* Lipid II are required for reconstitution of *S. aureus* PBPs' TP activities.

As mentioned in Chapter 1.5, the Kahne lab has developed a new synthetic route to achieve Lipid I, which involves independent preparations of the conserved *N*-acetyl-muramyl-pyrosphopho-lipid (MurNAc-PP-lipid) precursor and the peptide moiety.[8] The synthetic Lipid I can then be quantitatively converted to Lipid II using the MurG chemoenzymatic reaction.[9,10] It has been shown previously that the heptaprenyl-Lipid II (C35) analogue has better solubility than the native undecaprenyl-Lipid II (C55), hence is a better substrate for studying PBP activities *in vitro*.[11] Hiro Tsukamoto, a former postdoctoral researcher in the Kahne lab, has synthesized approximately 80 mg of the MurNAc-PP-hetaprenyl precursor after a 22-step synthesis for future preparations of Lipid I and Lipid II variants. With the precursor available, the challenge with chemical synthesis of bacterial Lipid II now shifts to obtaining various peptide moieties since the identity of the peptide is distinct in each organism. Therefore, for each new substrate of interest, the peptide portion needs to be synthesized first prior to coupling to the conserved MurNAc-PP-hetaprenyl precursor to yield Lipid I. Using this general synthetic route, three different Lipid I substrates have been prepared and converted to the corresponding Lipid II variants, including

Lys-Lipid II (the Lipid II precursor in Gram-positive bacteria), *m*-Dap-Lipid II (*E. coli* Lipid II), and amidated *m*-Dap-Lipid II (*B. subtilis* Lipid II).[8,12,13]

In 2012, we set out to apply this general synthetic route to prepare native *S. aureus* Lipid II that contains both a carboxamide and a pentaglycine branch in the peptide, as well as its monoglycine- and triglycine- variants. Having access to these Lipid II analogues would allow us to investigate the substrate specificity of *S. aureus* PBPs in the transpeptidation reaction. In this chapter, my efforts to synthesize three glycine-variants of L-Ala-D-*iso*Gln-L-Lys-D-Ala-D-Ala pentapeptide (in short, Gly-pentapeptide, Gly_3-pentapeptide and Gly_5-pentapeptide) were discussed. While small quantities of Gly-Lipid II and Gly_3-Lipid II substrates were obtained via this synthetic route, the synthesis was inefficient and had low overall yield.

On the other hand, we established a direct Lipid II accumulation and isolation strategy that came about from our studies with *S. aureus* PBP4 described in Chapter Two. We show that useful quantities of native *S. aureus* Lipid II can be obtained over a day of work, circumventing the need for laborious synthesis. We also demonstrated that the strategy is suitable for obtaining Lipid II from other bacteria as well, including *S. aureus* Δ*femA* and Δ*femB* mutants, *Bacillus subtilis* (*B. subtilis*), *Enterococus faecalis* (*E. faecalis*) and *Escherichia coli* (*E. coli*). The Lipid II substrate provides essential tools for biochemical reconstitution of *S. aureus* PBP2 TP activity described in Chapter Four.

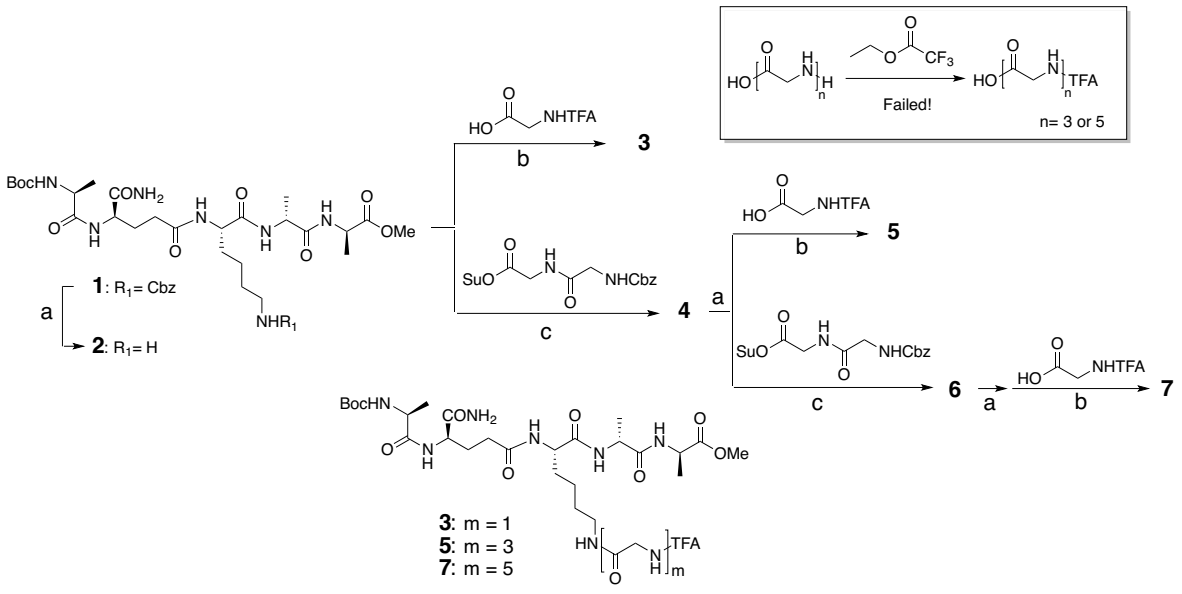
3.2 Chemical synthesis of *S. aureus* Lipid II

3.2.1 Synthesis of stem pentapeptide containing variable glycine branch

In the synthetic scheme to Lipid I, the highly acid-sensitive pyrophosphate linkage that is both glycosidic and allylic is introduced into the MurNAc-PP-lipid precursor separately from the

synthesis of the peptide.[8] Nevertheless, the peptide moiety should still be appropriately protected with base-labile protecting groups that are compatible with the pyrophosphate linkage. As with previous Lipid I and Lipid II syntheses, we chose a methyl ester group to protect the carboxylate end of the D-Ala-D-Ala terminus in the stem pentapeptide,[8,12,13] and a trifluoroacetic acid (TFA) group to mask the nucleophilic amine at the N-terminus of the glycine branch. Both protecting groups were installed during peptide synthesis and could be subsequently removed after Lipid I coupling in a global deprotection reaction. With the need to install different versions of the glycine branch onto the stem peptide, we used solution-phase synthesis to prepare the peptide moiety. Following a similar scheme reported by Tsukamoto and Kahne (2011),[8] we prepared the linear stem pentapeptide (**1**), Boc-L-Ala-D-*iso*Gln-L-Lys(Cbz)-D-Ala-D-Ala-OMe using standard peptide coupling synthesis. However, the presence of an unprotected carboxamide in the stem peptide led to several undesirable outcomes, including slow and incomplete coupling reaction and low yield of the product. Alternative attempts to avoid the complications of the unprotected carboxamide in peptide synthesis are discussed below. Hydrogenation of **1** revealed the ε-amine of the L-Lys side chain in **2** for attachment of the glycine branch. The Gly-pentapeptide (**3**), Boc-L-Ala-D-*iso*Gln-L-Lys(-GlyTFA)-D-Ala-D-Ala-OMe was afforded in a reaction with **2** and the commercially available TFA-Gly-OH (Scheme 3.1). We envisioned a parallel approach to yield the Gly_3- and Gly_5-pentapeptide variants. However, preparations of TFA-Gly_3-OH and TFA-Gly_5-OH from commercial reagents H-Gly_3-OH and H-Gly_5-OH using ethyl trifluoroacetate were not successful due to the insoluble nature of the oligoglycines. Thus, direct installations of the Gly_3- and Gly_5-branch onto **2** were not possible. In particular, Patin *et al*. encountered similar difficulties during pentaglycine synthesis, and turned to an iterative coupling approach instead.[4] As a result, we revised the synthetic route to add glycines onto **2** in a stepwise manner. As shown

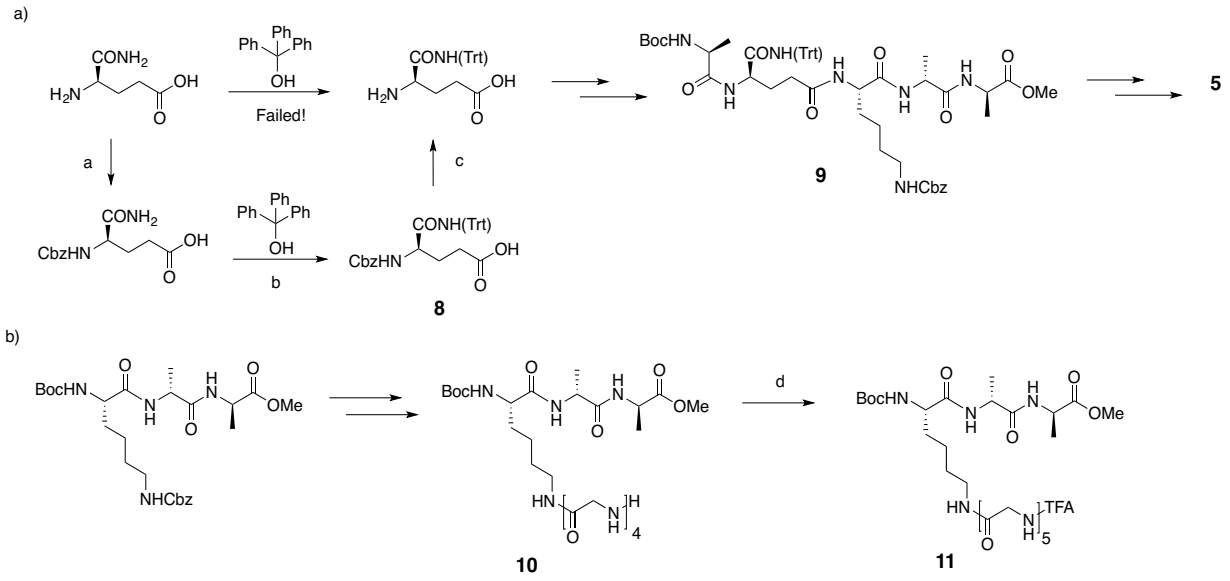
in Scheme 3.1, the stem pentapeptide (**2**) was first coupled with an appropriately protected *N*-succinimidyl ester activated diglycine, Cbz-Gly-Gly-OSu (Cbz is caboxybenzyl) to afford intermediate (**4**), followed by hydrogenation and then addition of TFA-Gly-OH to yield the Gly_3-pentapeptide (**5**), Boc-L-Ala-D-*iso*Gln-L-Lys(-GlyGlyGlyTFA)-D-Ala-D-Ala-OMe. To make the Gly_5-pentapeptide (**7**), Boc-L-Ala-D-*iso*Gln-L-Lys(-GlyGlyGlyGlyGlyTFA)-D-Ala-D-Ala-OMe, the coupling of Cbz-Gly-Gly-OSu and the subsequent hydrogenation were performed twice successively, prior to the final attachment of TFA-Gly-OH. However, this strategy had several notable disadvantages. First, the coupling of Cbz-Gly-Gly-OSu became less efficient as the glycyl branch grew longer, requiring extended reaction time. Unfortunately, prolonged incubation under basic aqueous solution subjected the methyl ester protecting group on the stem pentapeptide to potential hydrolysis. In addition, purification of the product from the solution mixture was problematic. The insolubility of the glycyl-peptides defied traditional work-up using common organic solvents. Therefore, I loaded the reaction mixture (in water) onto a C-18 solid phase extraction column, and washed the column with a step gradient of acetonitrile and water mixture. Although this purification method afforded low amount of the pure peptide product, the recovery yield was significantly reduced (approximately 20% recovery yield). Given that two successive coupling and deprotection steps were required for the synthesis of **7**, this strategy deemed considerably inefficient and tedious. Furthermore, the final step in the syntheses of both **5** and **7** was the addition of TFA-Gly-OH. This reaction was also very sluggish, requiring multiple rounds of addition of excess reagents to facilitate reaction progression. In the case of **7**, the coupling was incomplete after a 72-hour reaction with approximately 20-equivalent of the TFA-Gly-OH added. Overall, only less than 10 mg of **7** was obtained by combining the products from several rounds of syntheses after a few months of work.



Scheme 3.1. Synthetic route of Gly-pentapeptide (**3**), Gly_3-pentapeptide (**5**), Gly_5-pentapeptide (**7**). As shown in the box, trifluoroacetylation of the N-terminal amine in H-Gly-Gly-Gly-OH and H-Gly-Gly-Gly-Gly-Gly-OH did not work due to insolubility of the oligoglycine starting materials. Thus, a direct coupling of the oligoglycine branch to the stem pentapeptide was not possible; instead, an iterative method was taken. a: H_2, $Pd(OH)_2$/C, MeOH; b: TFA-Gly-OH (5-10 equivalent), HOBt, EDC, DIEA, DCM/DMF, r.t. 12-72 h; c: Cbz-Gly-Gly-OSu (2-5 equivalent) 1M $NaHCO_3$, DME, r.t., 2-12 h.

To improve the synthetic efficiency of the glycyl-pentapeptide compounds, I attempted several modifications of the synthetic route, which are summarized here. As mentioned earlier, the presence of an unprotected carboxamide is known to cause complications in peptide synthesis, such as slow, incomplete coupling reactions and by-product (i.e. pyroglutamate) formation. In addition, peptides with an unprotected carboxamide often display low solubility and aggregation due to hydrogen bonding.[14] In this regard, I sought to protect the carboxamide with a triphenylmethyl group, which was used in the previous synthesis of *B. subtilis* Lipid II which contains an amidated *m*-Dap residue.[13] Since the D-*iso*Gln(Trt)-OH reagent was prohibitively expensive, I had to prepare it in-house following tritylation protocols. The direct tritylation of D-*iso*Gln-OH by triphenylmethanol did not work, possibility due to the low

solubility D-*iso*Gln-OH and the potential formation of a pyroglutamate as reported by Sieber and Riniker (1991).[14] Therefore, I used an indirect tritylation protocol instead: first protecting the N-terminal amine on D-isoGln-OH with a Cbz group prior to tritylation to afford Cbz-D-*iso*Gln(Trt)-OH, followed by hydrogenation to yield the desired D-*iso*Gln(Trt)-OH (Scheme 3.2a). However, additional protection and deprotection steps were introduced for tritylation in this manner, and the overall yield was far from optimal (18%). As a proof of concept, the trityl-protected pentapeptide (**9**), Boc-L-Ala-D-*iso*Gln(Trt)-L-Lys(Cbz)-D-Ala-D-Ala-OMe was prepared, to which a triglycine branch was appended in the stepwise fashion as discussed above (Scheme 3.2a). Masking the carboxamide with a trityl protecting group indeed improved the solubility of the intermediates and eased the purification steps, and the trityl group was readily removed with TFA. Using this route, the Gly_3-pentapeptide (**5**) was obtained. Conceivably, it could be used to prepare the Gly_5-pentapeptide (**7**) as well. On the other hand, another route that involves the introduction of the carboxamide at the late step in the synthesis was attempted. The pentaglycine branch was installed onto the tripeptide precursor Boc-L-Lys-D-Ala-D-Ala-OMe to afford **11** using the similar sequential reactions (Scheme 3.2b).

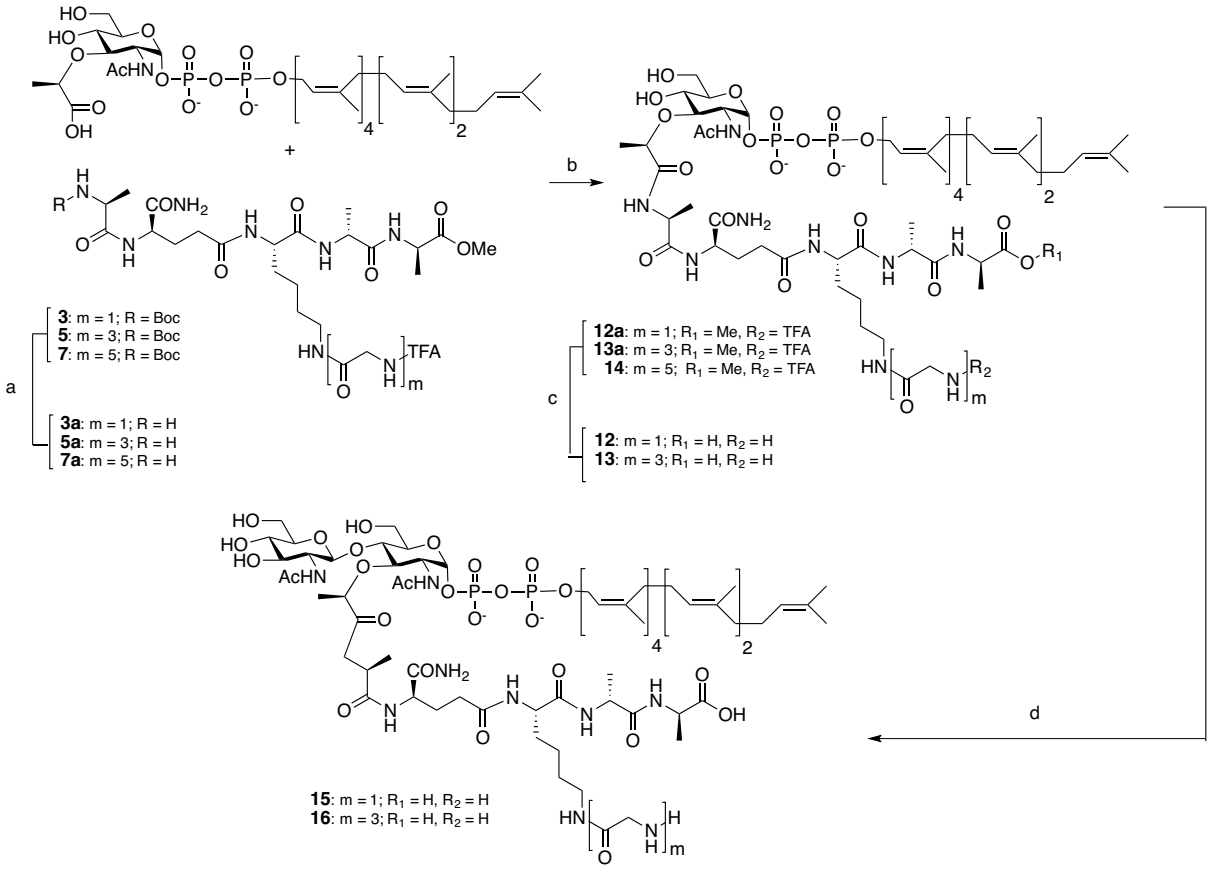


Scheme 3.2. Alternative routes attempted to avoid complication by the carboxamide in the peptide. In a), a trityl protecting group was installed to protect the carboxamide in D-*iso*Gln-OH in the early step of the synthesis. As shown, a direct triylation did not work, instead reactions a-c were performed. The D-*iso*Gln(Trt)-OH was used to make the stem pentapeptide, followed by the attachment of the glycyl branch. a: benzyl chloroformate, r.t. 12 h; b: triphenylmethanol, acetic anhydride, conc. sulfuric acid, glacial acetic acid; c: H_2, $Pd(OH)_2$/C, MeOH. In b), the glycyl branch was coupled to the tripeptide Boc-L-Lys-D-Ala-D-Ala-OMe to ensure introduction of the carboxamide in a late step in synthesis.

3.2.2 Coupling reactions to make Lipid I and Lipid II variants

To obtain Lipid I, the MurNAc-PP-lipid precursor was coupled with the respective peptide moiety, **3**, **5** or **7** using the dimethoxytriazine-N-methylmorpholinium chloride (DMTMM) as a coupling reagent (Scheme 3.3).[8] Because of difficulties in obtaining large amount of the peptide and the limited availability of the MurNAc-PP-lipid precursor, this reaction was usually performed in small scale using 3-5 mg of the MurNAc-PP-lipid only. The reaction progression was monitored by the appearance of the corresponding Lipid I peak on MS. In these coupling reactions, Gly-Lipid I and Gly_3-Lipid I products were obtained, but the Gly_5-Lipid I was detected at a low level after prolonged reaction. While the amount of Gly_5-pentapeptide (**7**) diminished in the reaction over time, there was still an excess of the unreacted

MurNAc-PP-lipid, indicating possible degradation of **7** to result in incomplete reaction. However, the Gly_5-Lipid I might be difficult to detect on MS since it ionizes poorly, as reported by Schneider *et al.*[3] Upon purification of the reaction, I obtained approximately 1 mg each for the protected Gly-Lipid I and Gly_3-Lipid I products. To remove protecting groups on the peptide moiety, the protected Lipid I was subject to hydrolysis in a basic solution. The base hydrolysis reaction should be closely monitored since long incubation time (more than 5 h) may result in cleavage of the glycine branch. Gly-Lipid I (**12**) and Gly_3-Lipid I (**13)** were obtained in small quantities. Subsequently, Lipid I was converted to Lipid II using the MurG chemoenzymatic reaction with a recovery yield of approximately 50%. We note that our *in vitro* reaction sequence is the opposite of the *in vivo* sequence in *S. aureus*, where MurG reaction occurs prior to the addition of the glycyl branch by FemXAB proteins.[15,16] Hence the Gly-Lipid I and Gly_3-Lipid I substrates might not be the optimal substrates for MurG *in vitro*, limiting the conversion yield. Small amounts of Gly-Lipid II (**15**) and Gly_3-Lipid II (**16**) were yielded from these reactions.



Scheme 3.3. The general reaction sequence to afford Lipid I and Lipid II variants. a: 4 M HCl in dioxane; b: DMTMM, DIEA, MeOH; c: 1M LiOH, THF/H_2O; d: MurG, UDP-GlcNAc.

3.3 A facile method to obtain native *S. aureus* Lipid II

Given the challenges encountered with the chemical synthesis of the pentaglycine containing Lipid II, we sought an alternative strategy to obtain the substrate. As described in Chapter Two, we developed a simple and sensitive Western blot assay to detect the cellular levels of Lipid II in bacteria.[17] In that study, we observed that moenomycin treatment in *S. aureus* accumulates Lipid II in cells. Moenomycin is a natural product inhibitor that targets bacterial PGTs to prevent monomeric Lipid II from being polymerized,[18] hence accumulating

Lipid II. Thus, we wondered whether it would be possible to obtain enough Lipid II substrate by directly isolating from moenomycin-treated *S. aureus* culture.

To better characterize Lipid II accumulation caused by moenomycin, we performed a time course analysis to monitor levels of Lipid II in *S. aureus* after moenomycin treatment. Consistent with the bacteriostatic effect of moenomycin in Gram-positive bacteria,[19] *S. aureus* growth stopped immediately after the addition of moenomycin to the culture (Figure 3.1a). At the indicated time point (15 min, 60 min, 120 min), an aliquot (1 mL) was collected from either untreated or moenomycin-treated *S. aureus* culture respectively, and was subjected to Lipid II analysis using previously described protocol.[17] As shown in Figure 3.2b, the level of Lipid II in *S. aureus* increased within 15 min of moenomycin treatment and remained elevated for at least 2 h. The amount of Lipid II in *S. aureus* increased by approximately 10-fold after treating with moenomycin for 15 min (Figure 3.1c).

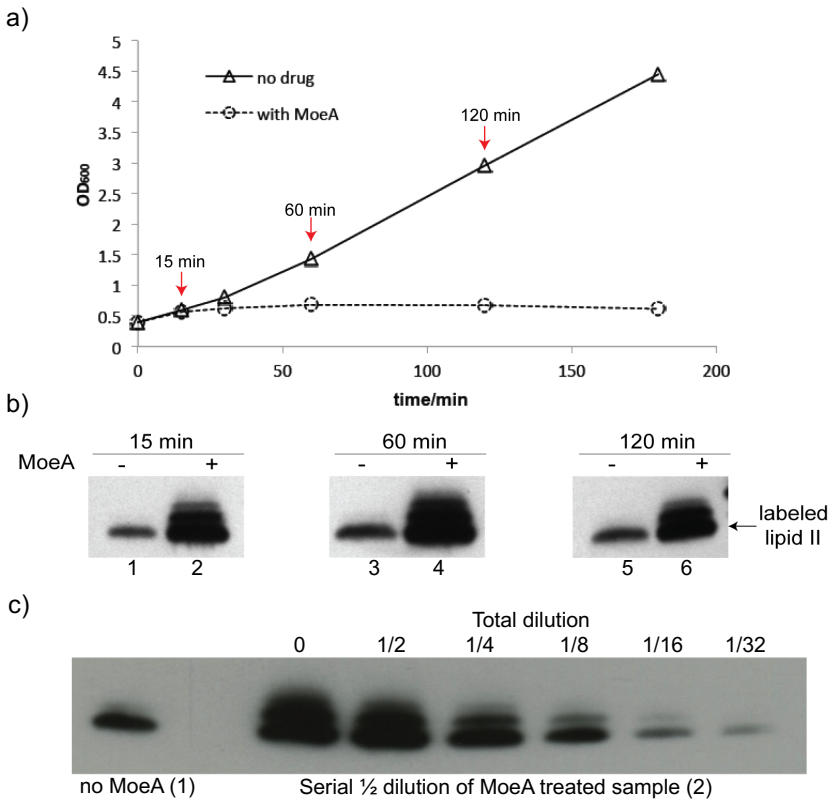


Figure 3.1. Moenomycin treatment accumulates Lipid II by 10-fold in *S. aureus*. (a) Growth curves of untreated and moenomycin-treated *S. aureus*. An aliquot from each culture was collected at the indicated time for analysis in b. (b) Western blot analysis of biotinylated-Lipid II in each sample. The Lipid II level increased within 15 min of moenomycin treatment and remained elevated for at least 2 h. (c) A serial dilution estimation that Lipid II level increased 10-fold after treating *S. aureus* with moenomycin for 15 min.

The strong and stable accumulation of Lipid II observed in moenomycin-treated *S. aureus* culture gave us a window to perform a large-scale Lipid II extraction. We treated 6 L (4 x 1.5 L) of *S. aureus* treated with moenomycin (0.6 μg/mL) for 20 minutes, pelleted down cells, and extracted the cellular lipids with $CHCl_3$/MeOH. In a large-scale extraction, the total volume of organic solvent used per cell culture volume was less than that in the previous small-scale extraction, giving rise to more concentrated cellular lipids. We observed a white emulsion in the interfacial layer between the aqueous and organic layers at the end of the extraction, which was

not observed in the small-scale extraction. To analyze contents in the interface, we collected the interface separately from the organic layer by pipetting out each layer. As shown in the thin layer chromatography (TLC) analysis in Figure 3.2b, the majority of phospholipids were found in the organic layer but little was present in the interface. We next evaluated the presence of Lipid II in the interface and organic layers by PBP4-enabled biotinylation and Western blot. Surprisingly, in contrast to phospholipids, Lipid II was concentrated in the interface layer. The formation of the third interface layer that contains Lipid II and is clean of abundant phospholipid contaminants was intriguing. We hypothesized that the amount of accumulated Lipid II in large-scale extraction exceeded its solubility in the organic phase, giving rise to an interfacial layer between the aqueous and organic fractions. In a previous report Guan *et al*., *E. coli* Lipid II was observed to partition into the aqueous phase at neutral pH during extraction.[20] The authors took advantage of the unusual pH-dependent solubility of *E. coli* Lipid II to enrich for Lipid II from the abundant phospholipids. Our observation of the third interfacial layer in large-scale extraction of *S. aureus* also enabled us to selectively enrich *S. aureus* Lipid II. Therefore, we collected the interface as the source of Lipid II.

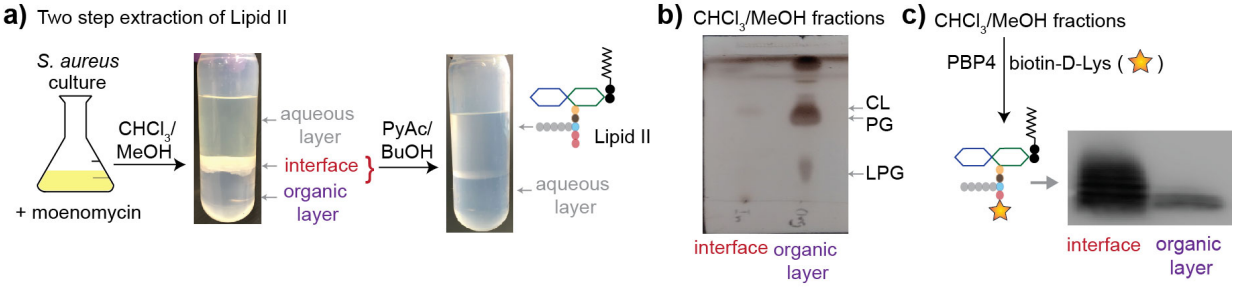


Figure 3.2. Preparative quantities of native *S. aureus* Lipid II can be obtained via a two-step accumulation and extraction procedure. (a) Schematic of the extraction procedure. White materials at the interface were observed after $CHCl_3$/MeOH extraction from an *S. aureus* culture treated with moenomycin. The interface was collected and further extracted with pyridinium acetate/n-butanol to obtain Lipid II in the organic layer. (b) Thin layer chromatography showed that phospholipids are predominantly in the organic layer after $CHCl_3$/MeOH extraction. (CL: cardiolipin; PG: phosphatidylglycerol; LPG: lysyl-phosphatidylglycerol) (c) Western blotting showed that Lipid II is predominantly in the interface layer after $CHCl_3$/MeOH extraction.

In order to establish the structure of the Lipid II species in the interface, we cleaved the pyrophosphate linkage by boiling the material in a weakly acidic buffer.[a] After mild acid hydrolysis,[21,22] the sample was subjected to MS analysis. As expected, we observed a peak corresponding to the monophosphate muropeptide, which is the cleaved product of *S. aureus* Lipid II. However, a major peak corresponded to the Park nucleotide was present as well (Figure 3.3). It is known that many cell wall-targeting antibiotics including moenomycin can result in accumulation of the Park nucleotide in bacteria.[23,24] As described in Chapter 1.2, the observation that the Park nucleotide accumulates in *S. aureus* as a result of penicillin treatment led to the initial discovery of the cell wall pathway.[25] Since the Park nucleotide is a precursor of Lipid II and its peptide portion closely resembles that of Lipid II, we reasoned that the Park nucleotide should be removed from the sample to avoid any complications in using *S. aureus* Lipid II as a substrate. Therefore, we performed an additional extraction of the interface layer using a mixture of 6 M pyridinium acetate and n-butanol (Figure 3.2). This solvent system was initially reported by Strominger and coworkers for Lipid II extraction.[26,27] In this step, *S. aureus* Lipid II selectively partitioned into the organic phase, while the soluble Park nucleotide was in the aqueous phase. We collected the organic fraction that contains *S. aureus* Lipid II. The structural assignment of the delipidated Lipid II after mild acid hydrolysis was confirmed by LC/MS/MS analysis. The species contains a pentaglycine branch and a carboxamide in the stem peptide; we did not detect any related species such as the ones lacking some of the glycines or the carboxamide. TLC analysis of the isolated *S. aureus* Lipid II showed one major spot, which has comparable R_f value as synthetic Lys-Lipid II (Figure 3.3b). Therefore, we conclude that native *S. aureus* Lipid II was obtained from *S. aureus* by direct isolation.

[a] Since direct injection of the sample did not yield any mass corresponding to Lipid II, we reasoned that

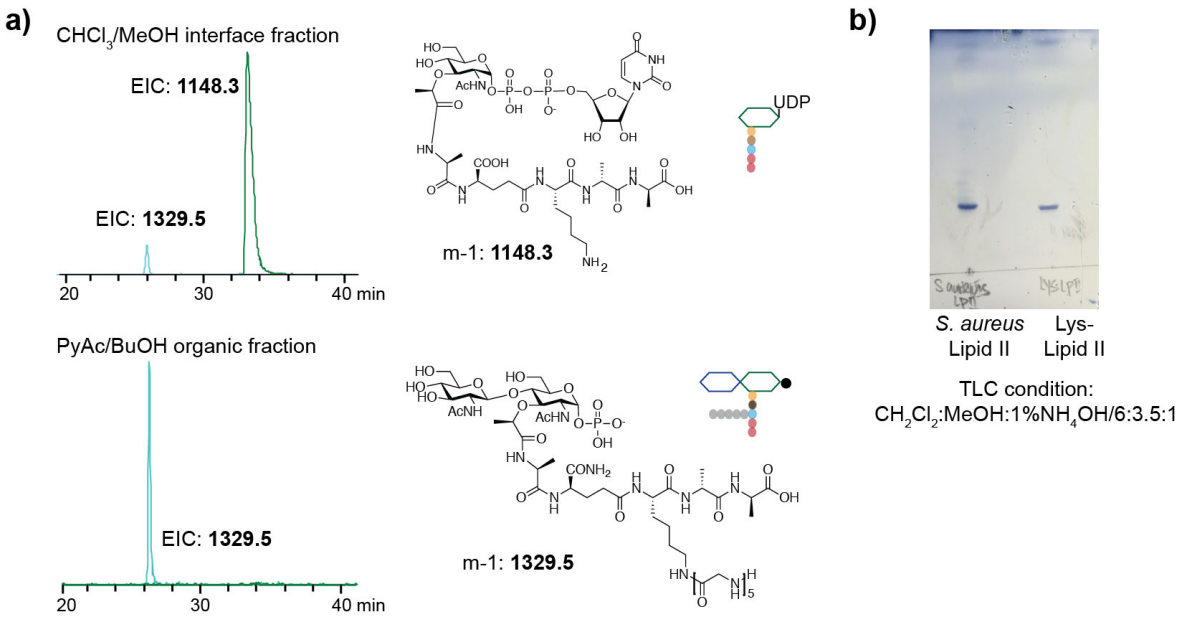


Figure 3.3. Structural confirmation of the isolated *S. aureus* Lipid II. a) EICs showing both delipidated *S. aureus* Lipid II and the Park nucleotide were detected in the interface (top), and only deplipidated *S. aureus* Lipid II was observed in the organic phase after the additional extraction using pyridinium acetate/n-butanol (bottom). The samples were boiled in a weak acidic buffer prior to LC/MS analysis. The structures of the species are shown. b) TLC analysis of the isolated *S. aureus* Lipid II and synthetic Lys-Lipid II.

3.3.1. Estimation of *S. aureus* Lipid II quantity

In order to determine the amount of *S. aureus* Lipid II obtained by extraction, we used synthetic Lys-Lipid II as a standard for estimation. We subjected a mixture of Lys-Lipid II and *S. aureus* Lipid II to mild acid hydrolysis for cleavage of the pyrophosphate linkage, and then compared the ion counts of muropeptide **1** (from the synthetic Lys-Lipid II) and muropepitde **2** (from *S. aureus* Lipid II) on LC/MS (Figure 3.4). A standard curve was made based on the counts of muropeptide **1** at different concentrations, and was used to calculate the concentration of muropeptide **2**. It was estimated that approximately 300 μg of *S. aureus* Lipid II was obtained from 1 L of moenomycin-treated *S. aureus* culture. In addition, an orthogonal quantification

method to determine the amount of *S. aureus* Lipid II was performed. Densitometry analysis of the biotinylated synthetic Lys-Lipid II and *S. aureus* Lipid II led to approximately 800 μg of *S. aureus* Lipid II from 1 L of moenomycin-treated *S. aureus* culture (Figure App2.22). While the quantitative measurement of *S. aureus* Lipid II extracted is still ongoing, we note that the estimated amount of Lipid II is sufficient for a large number of enzymatic assays (see Chapter Four). Taken together, useful quantities of native *S. aureus* Lipid II was obtained by direct isolation from moenomycin-treated *S. aureus* culture. This method can be accomplished in one day of work without the need for special equipment or synthetic expertise, greatly expanding the accessibility to the complex molecules.

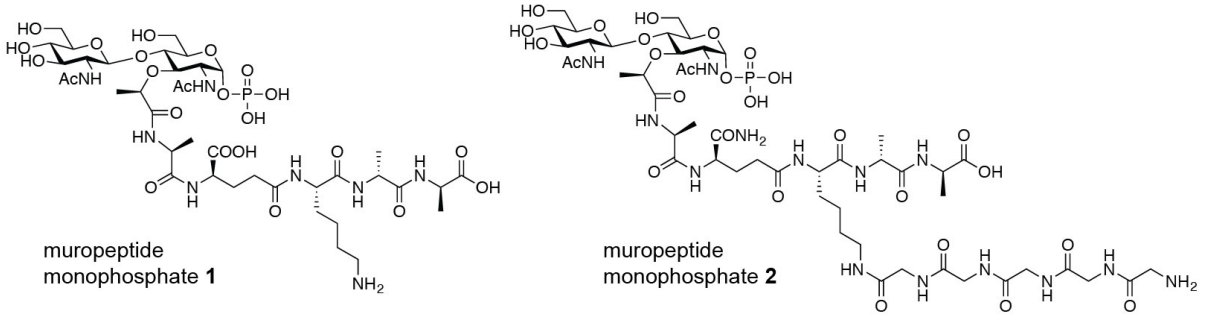


Figure 3.4. Structures of delipidated Lipid II species. The species were yielded after acid hydrolysis of synthetic Lys-Lipid II (**1**) and *S. aureus* Lipid II (**2**).

3.4 A general approach to obtain bacterial Lipid II

In contrast to chemical syntheses of Lipid II, where a new synthetic route needs to be developed for each new Lipid II variant, our accumulation and isolation strategy presents a universal solution to obtain native Lipid II from different bacteria. Up to date, we have demonstrated the utility of this approach for a number of strains, including *S. aureus* Δ*femA* and Δ*femB* mutants, *B. subtilis, E. faecalis* and *E. coli*. Since *B. subtilis* is intrinsically resistant to

moenomycin,[b] we chose vancomycin for Lipid II accumulation. In the case of *E. coli*, a Gram-negative bacteria which has an impermeable outer membrane that presents an entry barrier to both moenomycin and vancomycin antibiotics, we resorted to a mutant *E. coli* strain (*Imp*), which has an defective outer membrane and restores the susceptibility to both antibiotics for Lipid II extraction. In addition, blocking the Lipid II flippase, MurJ with (2-sulfonatoethyl)methanethiosulfonate (MTSES) in a $MurJ^{A29C}$ mutant strain of *E. coli*[28,29] provides an alternative route to accumulate Lipid II. Notably, an interfacial layer was obtained during large-scale Lipid II extraction for each strain examined, suggesting the formation of the interface containing concentrated Lipid II is a common phenomenon.

We analyzed the isolated Lipid II by *S. aureus* PBP4-enabled BDL labeling, and found that short glycan polymers (of approximately 3-5 disaccharide repeats) in addition to Lipid II monomers were accumulated in *B. subtilis* as a result of vancomycin treatment. This observation reflects a possible mechanism of vancomycin in sequestering both short nascent PG polymers as well as Lipid II in *B. subtilis*. Nevertheless, the short glycan polymers could be completely removed by treating the interface sample with mutanolysin. Furthermore, we determined the structure of the isolated Lipid II species by LC/MS after mild acid hydrolysis. We confirmed that Gly-Lipid II and Gly_3-Lipid II were obtained from *S. aureus* Δ*femA* and Δ*femB* mutants respectively, and both substrates were used in biochemical studies of *S. aureus* PBP2 in Chapter Four. Isolated Lipid II from *B. subtilis* was identified to contain an amidated *m*-Dap in the stem peptide, whereas Lipid II from *E. faecalis* showed an L-Ala-L-Ala branch with either a carboxamide or a carboxylate at the second position of the stem peptide (Figure 3.5 and Figure App. 2.23). The two species of *E. faecalis* Lipid II were present in comparable quantities. The

[b] Treatment of *B. subtilis* with a sub-lethal concentration of moenomycin still leads to an accumulation of Lipid II.

amidation state on Lipid II in certain bacteria may vary with the growth phase of the organism.[30] For instance, Lipid II species in *mycobacteria* is highly heterogeneous with multiple modifications;[22] however, the heterogeneity in *E. faecalis* Lipid II has not been reported previously.

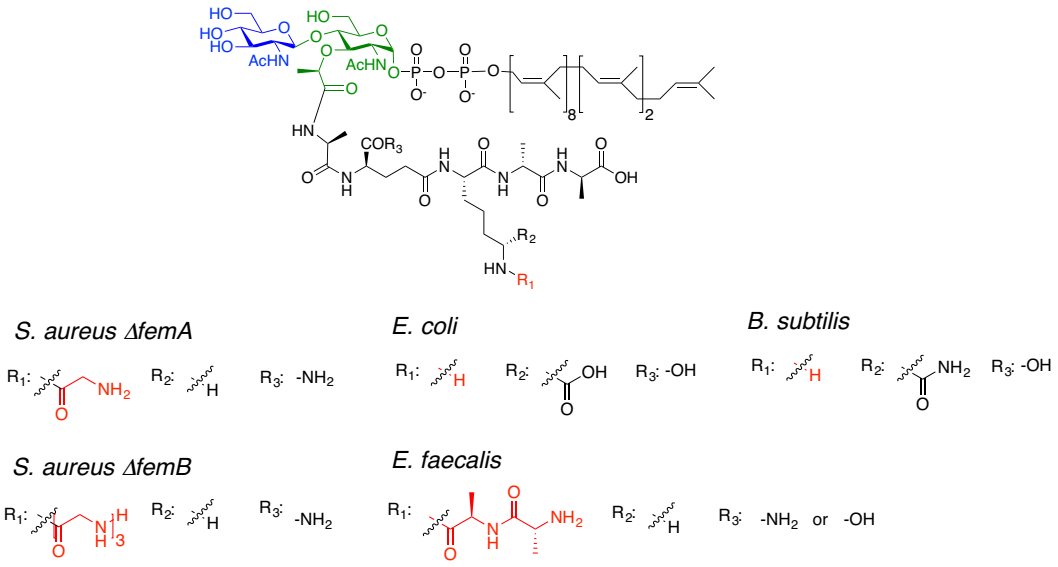


Figure 3.5. Structures of Lipid II species isolated from different bacteria. The Lipid II accumulation and extraction protocol was used.

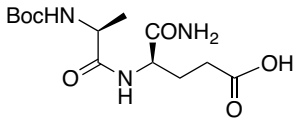
3.5 Conclusion

In summary, the chemical synthesis of *S. aureus* Lipid II faced significant challenges due to insolubility of the pentaglycine branch and the D-*iso*Gln in the stem peptide. Although Lipid II variants with mono- and tri-glycine branch were made, the overall yields were low. Instead, we developed a facile extraction method to obtain native *S. aureus* Lipid II from moenomycin-treated *S. aureus* culture. Previously, direct isolation of Lipid II from bacteria has been considered impractical due to low levels (2000 molecules/cell) of Lipid II in bacteria.[2] However, our studies showed that treatment of moenomycin in *S. aureus* led to a 10-fold increased in Lipid

II relative to untreated *S. aureus*, providing an opportunity for its direct extraction. In the large-scale Lipid II extraction, we noted that the accumulated Lipid II concentrated at an interface layer, allowing its effective enrichment and removal from the abundant phospholipids. The interface was collected and extracted with organic solvents to remove the Park nucleotide and afforded the relatively pure Lipid II. The isolated *S. aureus* Lipid II contains both a pentaglycine and a carboxamide in the peptide portion. Our work represents the first preparation of native *S. aureus* Lipid II. We estimated that 500 μg[c] of *S. aureus* Lipid II can be obtained from 1 L of moenomycin-treated *S. aureus* culture, sufficient for more than 1000 biochemical assays described in Chapter Four. In addition, this strategy is also suitable for obtaining Lipid II from other bacteria, including *S. aureus* mutants, *B. subtilis*, *E. faecalis* and *E. coli*. This direct isolation approach to yield Lipid II can be accomplished over a day without the need of expensive starting materials and synthetic expertise, greatly lowering the barriers to obtain various bacterial Lipid II.

3.6 Materials and Methods

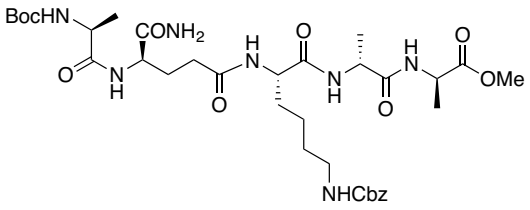
3.6.1 Experimental details of synthesis



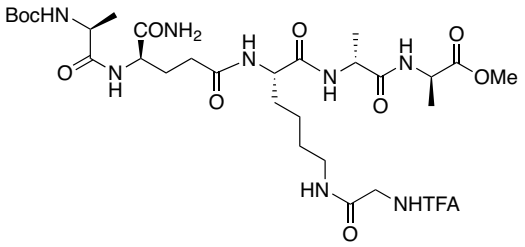
Boc-L-Ala-D-*iso*Gln-OH. To a stirring mixture of Boc-Ala-OSu (573 mg, 2.00 mmol) and H-D-Glu-NH_2 (293 mg, 2.00 mmol) in DME (6 mL) was added 1 M $NaHCO_3$ (12 mL) at room temperature. Vigorous stirring was needed to ensure proper mixing of the solids. After reaction for 15 h, the mixture was concentrated *in vacuo* until it became clear. The mixture was cooled at

[c] The average of the numbers from two estimation methods (see Chapter 3.3.1) was taken.

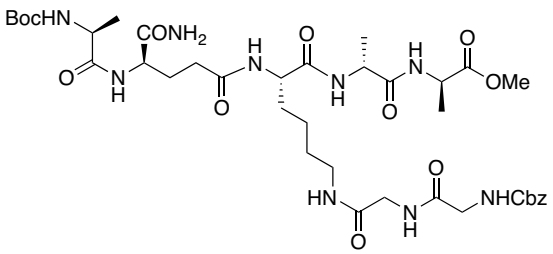
0 °C and acidified with 10 % aqueous citric acid (24 mL, 12 mmol). The mixture was extracted twice with EtOAc and the combined organic phases were washed with brine (12 mL), dried over Na_2SO_4, and then concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-OH as a white crystalline product (365 mg, 57.5%). δ_H (500 MHz; CD_3OD), 4.09 (1 H, d, *J* 7.1), 2.37 (2 H, d, *J* 7.5), 2.17-2.24 (1 H, m), 1.87-1.91 (1 H, m), 1.43 (9 H, s), 1.22-1.31 (3 H, m) (Figure App2.1).



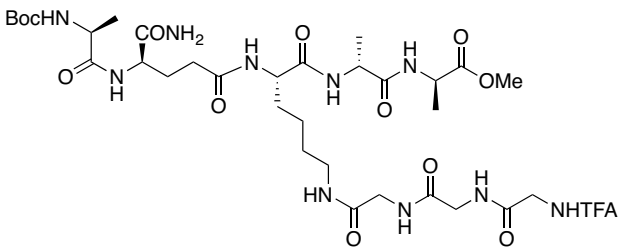
Boc-L-Ala-D-*iso*Gln-L-Lys(Cbz)-D-Ala-D-Ala-OMe (1). To a stirring solution of HCl NH_2-L-Lys(Cbz)-D-Ala-D-Ala-OMe (174mg, 0.37 mmol) in dry DCM (10 mL) was added Boc-L-Ala-D-*iso*Gln-OH (129 mg, 0.41 mmol), HOBt-H_2O (67.8 mg, 0.44 mmol), DIEA (77.2 µL), and EDC (84.9 mg, 0.43 mmol) at room temperature under N_2. After reaction for 7 h, the mixture was concentrated and partitioned between EtOAc (40 mL) and 10% citric acid (20 mL). A small amount of MeOH was added to enhance the solubility of the product. The organic phase was subsequently washed with H_2O (10mL), 5% $NaHCO_3$ (10mL), brine (10mL), and dried over Na_2SO_4, and concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-L-Lys(Cbz)-D-Ala-D-Ala-OMe as a yellow solid (231.6 mg, 85.4%). δ_H (500 MHz; CD_3OD) 7.34 (5 H, d, *J* 4.2), 5.06 (2 H, s), 4.35-4.40 (3 H, m), 4.01-4.20 (2 H, m), 3.68 (3 H, s), 3.12 (2 H, t), 2.25- 2.36 (2 H, m), 2.15-2.23 (2 H, m), 1.80-1.90 (2 H, m), 1.66-1.82 (2 H, m), 1.48-1.56 (2 H, m), 1.43 (s, 9H), 1.38-1.41 (3 H, d, *J* 7.3), 1.35-1.37 (3 H, d, *J* 7.3), 1.27-1.32 (3 H, d, *J* 7.3); LMRS (m/z) 736.3 (M+H) (Figure App2.2).



Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-TFA)-D-Ala-D-Ala-OMe (3). To a solution of **1** (120 mg, 0.22 mmol) in dry MeOH (15 mL) was added $Pd(OH)_2/C$ (30 mg). The mixture was let stir under N_2 for 20 min before applying a H_2 atmosphere at room temperature. After stirring under H_2 for 2 hr, the reaction mixture was filtered using a PDVF syringe filter. The filtrate was concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-L-Lys-D-Ala-D-Ala-OMe (**2**) (85 mg, quantitative) as a white solid. To dried **2** (20 mg, 0.03 mmol) in a clean RBF, TFA-Gly-OH (6.84 mg, 0.04 mmol), HOBt-H_2O (6.12 mg, 0.04 mmol) in a mixture of dry DCM/DMF (2 mL/0.5 mL) was added, followed by DIEA (7 µL, 0.04 mmol) and EDC (7.7 mg, 0.04 mmol). After the reaction was stirred at room temperature under N_2 for 15 h, the mixture was concentrated and resuspended in water (~ 2 mL) and purified using reverse phase C18 silica column using a step gradient: 0%, 10%, 25%, 50%, 75% acetonitrile (ACN)/ 0.1% formic acid (3 mL each). The product was eluted in 10% and 25% fractions, which were pooled and concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-TFA)-D-Ala-D-Ala-OMe (**3**) as a colorless film (9.7 mg, 50%). δ_H (500 MHz; CD_3OD) 4.35-4.40 (3 H, m), 4.19-4.22 (1 H, m), 4.03-4.05 (1 H, m), 3.91 (2 H, s, *J* 4.4), 3.70 (3 H, t, *J* 4.1), 3.21-3.23 (2 H, m), 2.34 (2 H, s), 2.20-2.23 (1 H, m), 1.86-1.88 (2 H, m), 1.69-1.70 (1 H, m), 1.54 (2 H, dd, *J* 4.3 and 2.8), 1.44-1.45 (9 H, m), 1.41 (3 H, ddd, *J* 7.1, 4.6 and 2.2), 1.37 (3 H, dd, *J* 6.4 and 3.6), 1.31-1.33 (3 H, m); LMRS (m/z) 755.3 (M+H) (Figure App2.3).

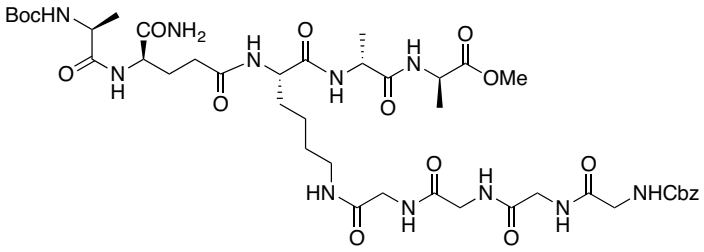


Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (4). To a stirred solution containing **2** (70 mg, 0.12 mmol) and Cbz-Gly-Gly-OSu (126.7 mg, 0.35 mmol) in DME (4 mL) and 1 M $NaHCO_3$ (12 mL) at room temperature. Vigorous stirring was necessary to ensure proper mixing of reagents. After the reaction was completed after stirring for 4 h, an excess amount of water was to dissolve the white solids before the mixture was concentrated *in vacuo*. The product was purified on reverse phase C18 silica using a step gradient: 0%, 10%, 10%, 20%, 30%, 40%, 60% ACN/ 0.1% formic acid (3 mL each). The 20% and 30% fractions were pooled and concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe **(4)** as a colorless film (57.4 mg, 56%). δH (500 MHz; CD_3OD) 7.29-7.39 (5 H, m), 5.12 (2 H, d, *J* 5.2), 4.35-4.40 (2 H, m), 4.20 (1 H, s), 4.05 (1 H, t, *J* 7.0), 3.84 (4 H, d, *J* 12.5), 3.69 (2 H, d, *J* 5.7), 3.20 (2 H, d, *J* 6.0), 2.35 (1 H, t, *J* 6.7), 2.20-2.21 (1 H, m), 1.86-1.89 (1 H, m), 1.75-1.80 (1 H, m), 1.66-1.72 (1 H, m), 1.54 (1 H, d, *J* 6.0), 1.42-1.45 (9 H, m), 1.42 (3 H, t, *J* 5.0), 1.38 (3 H, t, *J* 5.6), 1.33 (3 H, d, *J* 7.1); LMRS (m/z) 850.3 (M+H) (Figure App2.4).

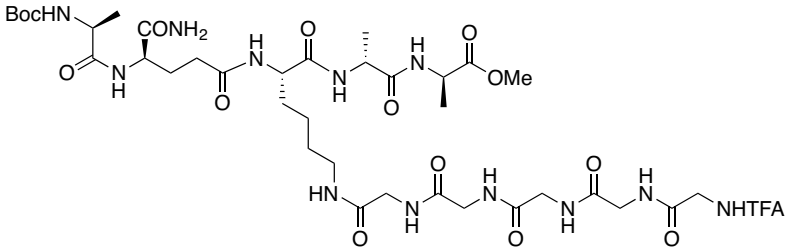


Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (5). To a mixture of **4** (50 mg, 0.059 mmol) in dry MeOH (10 mL) was added $Pd(OH)_2/C$ (10 mg). The mixture was stirred under N_2 for 10 min before applying an H_2 atmosphere for 2 h at room temperature. The mixture was filtered via a PVDF syringe filter (0.2 μM) and concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe a colorless film (40 mg, 95%). To Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (15 mg, 0.02 mmol), was added TFA-Gly-OH (10 mg, 0.06 mmol), HOBt-H_2O (4.8 mg, 0.03 mmol) and dry DCM (2 mL) as well as DMF (0.5 mL), followed by DIEA (5.8 μL) and EDC (6 mg, 0.03 mmol). The mixture was stirred under N_2 at room temperature for 30 h, during which more reagents: TFA-Gly-OH (10 mg), HOBt-H_2O (4.8 mg), DIEA (10 μL) and EDC (12 mg) were added. The progress of the reaction was monitored. After reaction was stirred for 48 h, 90% conversion was observed. The mixture was concentrated, and redissolved in 1: 1/H_2O: ACN (100 μL) to apply onto a C18 reverse phase column. The mixture was eluted using a step gradient: 0%, 10%, 20%, 30%, 40%, 50% ACN/0.1% formic acid. Unreacted starting material Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe was eluted in the 10% fraction; while the desired product Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe **(5)** was detected in the 20% fraction, which was concentrated *in vacuo* as a colorless film (3 mg, 16.4%). δ_H (500 MHz; CD_3OD) 4.39 (3 H, dd, *J* 7.3 and 3.3), 4.18-4.21 (1 H, m), 4.05 (1 H, m), 4.04 (2 H, s), 3.91 (2 H, s), 3.84 (2 H, s), 3.69 (3 H, s), 3.21 (2 H, d, *J* 6.5), 2.34 (2 H, d, *J* 6.9), 2.20 (1 H, s), 1.87-1.89 (1 H, m), 1.76-

1.80 (1 H, m), 1.67-1.70 (1 H, m), 1.53-1.56 (3 H, m), 1.44 (9 H, s), 1.41 (3 H, d, *J* 7.3), 1.37 (3 H, d, *J* 7.2), 1.32 (3 H, d, *J* 7.1); LMRS (m/z) 891.2 ($M+Na^+$) (Figure App2.5)

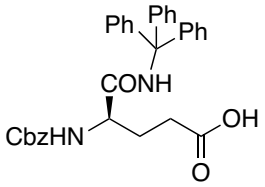


Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (6). To Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (40 mg, 0.056 mmol) in DME (2 mL) and 1 M $NaHCO_3$ (4 mL), was added Cbz-Gly-Gly-OSu (71.2 mg, 0.196 mmol) at room temperature. The progress of reaction was monitored. After the reaction was stirred for 30 h, more Cbz-Gly-Gly-OSu (50 mg, 0.14 mmol) was added. The reaction did not go to completion after 48 h, but significant amount of hydrolyzed products appeared. Therefore, the reaction was quenched by adding an excess amount of water and concentrated *in vacuo*. The mixture was purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%a, 20%b 30%a, 30%b, 40%, 60% ACN/ 0.1% formic acid (3 mL each). The 20% and 30% fractions were pooled and concentrated *in vacuo,* The 20%b and 30%a fractions were pooled and concentrated to afford Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**6**) as a colorless film (20 mg, 37%). δ_H (500 MHz; CD_3OD) 7.21-7.43 (5 H, m), 5.11-5.16 (2 H, s), 4.31-4.43 (3 H, m), 4.16-4.21 (1 H, m), 3.98-4.10 (2 H, m), 3.91-3.98 (2 H, m), 3.84-3.89 (4 H, m), 3.62-3.72 (3 H, m), 3.17-3.21 (2 H, m), 2.65-2.70 (1 H, m), 2.30-2.38 (2 H, m), 2.17-2.25 (2 H, m), 1.84-1.91 (2 H, m), 1.73-1.82 (2 H, m), 1.65-1.74 (2 H, m), 1.51-1.57 (2 H, m), 1.41-1.48 (9 H, m), 1.39-1.44 (3 H, d, *J* 7.1), 1.35-1.39 (3 H, d, *J* 7.1), 1.23-1.32 (3 H, d, *J* 7.1); LMRS (m/z) 964.4 (M+H) (Figure App2.6).



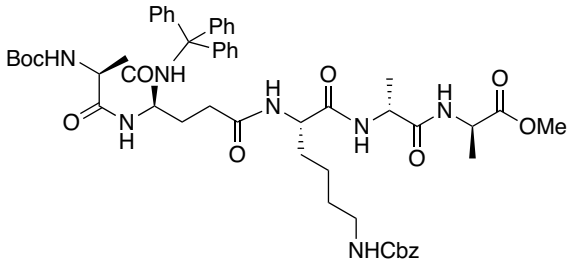
Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (7). To **6** (18.4 mg, 0.02 mmol) in dry MeOH (8 mL) was added $Pd(OH)_2/C$ (10 mg). The mixture was flushed with N_2 for 10 min before a H_2 atmosphere was applied for 2 h at room temperature. The mixture was filtered via a PVDF syringe filter (0.2 μM) and concentrated *in vacuo* to afford Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe as a colorless film (12.4 mg, 78%). To Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (12.4 mg, 0.015 mmol), was added TFA-Gly-OH (10 mg, 0.06 mmol), HOBt-H_2O (6.8 mg, 0.05 mmol) and dry DCM (2 mL) as well as DMF (0.5 mL), followed by DIEA (4.5 μL) and EDC (12 mg). The mixture was stirred under N_2 at room temperature for 72 h, during which more reagents: TFA-Gly-OH (10 mg, 0.06 mmol), HOBt-H_2O (6.8 mg, 0.05 mmol), DIEA (4.5 μL) and EDC (12 mg) were added. The reaction progress was monitored until starting material Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe was consumed. The mixture was concentrated, re-dissolved in a mixture of ACN/H_2O (200 μL) and purified on a C18 silica column with a step gradient: 0%, 5%, 10%a, 10%b, 20%a, 20%b, 30%a, 30%b, 40%, 60% ACN/0.1% formic acid (3 mL each). Unreacted starting material Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe was detected in 10%a and 10%b fractions, which were recovered; while the desired product Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe **(5)** was detected in 20%a fraction, which was concentrated *in vacuo* as a colorless film (5.1 mg, 34.7%). δ_H (500 MHz; CD_3OD) 4.35-4.41 (2 H, d), 4.25 (1 H, m), 4.17-4.21 (1 H, m), 4.16 (1 H, dd, *J* 2.5 and 1.5), 4.03 (2 H, dd, *J* 1.4 and 0.7), 3.91-3.94 (2 H, m), 3.88 (2H, m), 3.82-3.83 (2

H, m), 3.67 (2 H, m), 3.44 (2 H, s), 3.16 (2 H, t, *J* 1.6), 2.33 (2 H, t, *J* 0.8), 2.19 (2 H, m), 1.58-1.62 (3 H, m), 1.51-1.56 (2 H, m), 1.43-1.48 (9 H, m), 1.39-1.41 (2 H, m), 1.31-1.37 (4 H, m), 1.28-1.31 (3 H, m); LMRS (m/z) 983.3 (M+H) (Figure App2.7).



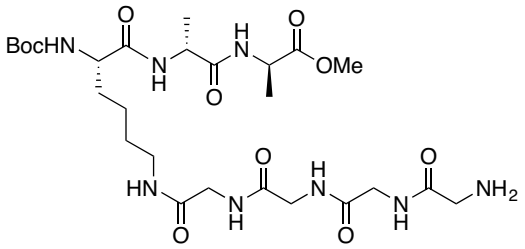
Cbz-D-*iso*Gln(Trt)-OH. To a stirring solution of D-Glu-NH_2 (670 mg, 4.6 mmol) in 1 M $NaHCO_3$ (1 mL) and H_2O (2 mL), was added benzylchloroformate (1.33 g, 7.8 mmol) dropwise. The mixture was vigorously stirred for 3.5 h at room temperature. The reaction was quenched with 10 mL H_2O. For purification, the crude mixture was extracted with diethyl ether (10 mL) for three times. The aqueous phase was collected, washed with 10% citric acid (10 mL), and extracted with EtOAc (20 mL). The organic phase was dried with Na_2SO_4, and concentrated *in vacuo* to afford Cbz-D-Glu-NH_2 a white powder (984 mg, 76%). LRMS (m/z): 279.1 (M-H). To the dried Cbz-D-Glu-NH_2 (192 mg, 0.685 mmol) was added triphenylmethnaol (375 mg, 1.37 mmol). To the solid mixture, glacial acetic acid (2 mL) was added, followed by the addition of 30 µL of conc. sulfuric acid in acetic acid (prepared by mixing 100 µL sulfuric acid and 1 mL acetic acid) dropwise. The mixture turned yellow upon addition. Acetic anhydride (129 µL, 1.3 mmol) was added lastly. The reaction was completed after stirring at room temperature for 2 h. Cold H_2O (20 mL) was added to quench the reaction, during which white solids were formed. The white solids were collected by filtration and purified on silica column chromatography using EtOAc/0.1% acetic acid and hexane/0.1% acetic acid as solvents. The Cbz-D-*iso*Gln(Trt)-OH eluted in 40% EtOAc/0.1% acetic acid, and concentrated to afford a yellow oil (153 mg, 43%).

δ_H (500 MHz; CD_3OD) 7.21-7.36 (20 H, mA), 5.22 (1 H, d, *J* 12.5), 5.05 (1 H, d, *J* 12.6,), 2.27 (2 H, dt, *J* 7.4 and 3.7), 1.98-2.00 (2 H, m), 1.97-2.00 (2 H, m); LMRS (m/z) 521.2 (M-H) (Figure App2.8).



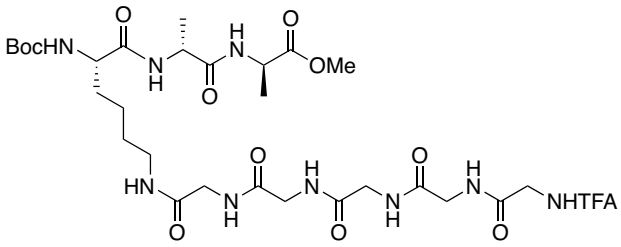
Boc-L-Ala-D-*iso*Gln(Trt)-L-Lys(Cbz)-D-Ala-D-Ala-OMe (9). To a stirring solution of Cbz-D-*iso*Gln(Trt)-OH (150 mg 0.287 mmol) in dry MeOH (25 mL) was added $Pd(OH)_2/C$ (60 mg). The mixture was stirred under N_2 for 10 min before applying H_2 atmosphere at room temperature. After reaction was stirred for 1 h, the reaction was filtered using a PVDF syringe filter and concentrated to afford H-D-*iso*Gln(Trt)-OH as a white solid (118.3 mg, 99%). To the vial containing deprotected H-D-*iso*Gln(Trt)-OH (87 mg, 0.342 mmol) in DME (4 mL) and 1 M $NaHCO_3$ (8 mL), was added Boc-L-Ala-OSu (87 mg, 0.342 mmol). After being stirred at room temperature for 24 h, the reaction was quenched with 10% citric acid (20 mL), and extracted with EtOAc (60 mL) twice. The organic layer was dried on Na_2SO_4, concentrated and purified using silica column chromatography. Boc-L-Ala-D-*iso*Gln-OH was eluted using EtOAc/0.1% acetic acid to afford a colorless liquid (90 mg, 47%). To a mixture of Boc-L-Ala-D-*iso*Gln(Trt)-OH (22 mg, 0.04 mmol) and H-L-Lys(Cbz)-D-Ala-D-Ala-OMe (437 mg, 0.04 mmol) in DCM (1.5 mL) and DMF (0.5 mL), was added HOBt-H_2O (6.61mg, 0.04 mmol), DIEA (10 μL, 0.04 mmol) and EDCI (8 mg, 0.04 mmol). The reaction was stirred at room temperature for 15 h. The mixture was concentrated, diluted with EtOAc (10 mL), washed with 10% citric acid (2 mL), the

organic phase was washed with H_2O (2 mL), 5% $NaHCO_3$ (1 mL), brine (1 mL), and dried over Na_2SO_4. The product was concentrated *in vacuo* and purified on silica column chromatography using EtOAc and hexane as eluent. Boc-L-Ala-D-*iso*Gln(Trt)-L-Lys(Cbz)-D-Ala-D-Ala-OMe was eluted in 100% EtOAc, and concentrated to afford a colorless oil (32.2 mg, 84%). δ_H (500 MHz; CD_3OD) 8.49 (1 H, s), 8.18-8.30 (2 H, m), 8.18 (1 H, d, *J* 7.3), 8.05 (2 H, d, *J* 7.5), 7.18-7.33 (16 H, m), 5.05 (2 H, s), 4.52 (1 H, ddd, *J* 5.0, 2.9 and 1.2), 4.33-4.38 (2 H, m), 4.14-4.16 (1 H, m), 4.04 (1 H, dd, *J* 6.8 and 2.0), 3.61 (3 H, s), 3.11 (2 H, t, *J* 6.1), 2.21-2.25 (3 H, m), 1.74-1.79 (2 H, m), 1.66-1.72 (1 H, m), 1.50 (2 H, d, *J* 6.8), 1.38-1.40 (9 H, m), 1.36 (3 H, d, *J* 3.3), 1.34-1.35 (3 H, m), 1.27 (3 H, t, *J* 9.5) (Figure App2.9).



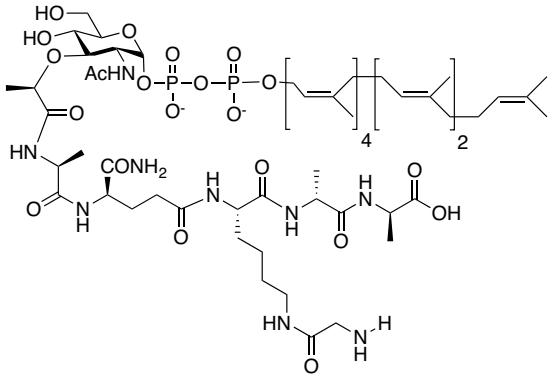
Boc-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (10). To a solution of Boc-L-Lys-D-Ala-D-Ala-OMe (137.2 mg, 0.34 mmol) in DME (2 mL) ad 1M $NaHCO_3$ (4 mL), was added Cbz-Gly-Gly-OSu (363.33 mg, 0.85 mmol). The mixture was stirred at room temperature for 4 h. The reaction was concentration *in vacuo* and dissolved in MeOH (3 mL). The Boc-L-Lys(-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe was purified on HPLC to afford a white solid (115.6 mg, 50%). To a solution of Boc-L-Lys(-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (115.6 mg, 0.18 mmol) in dry MeOH (8 mL), was added $Pd(OH)_2/C$ (40 mg). The reaction mixture was stirred under N_2 for 10 min before applying a H_2 atmosphere. After being stirred under H_2 for 2 h, the mixture was filtered using PVDF syringe filter and concentrated to afford Boc-L-Lys(-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe as a colorless film (89 mg, 96.8%). The Boc-L-Lys(-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (81

mg, 0.16 mmol) was stirred in DME (1.5 mL) and 1 M $NaHCO_3$ (3 mL), to which Cbz-Gly-Gly-OSu (142 mg, 0.39 mmol) was added. After the mixture was stirred at room temperature overnight, more Cbz-Gly-Gly-OSu (100 mg, 0. 27 mmol) was added with DME and 1 M $NaHCO_3$. The reaction was stirred for another 12 h before quenching with 10% citric acid. The mixture was concentrated *in vacuo* and purified on HPLC to afford Boc-L-Lys(-Gly-Gly-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe. Purified Boc-L-Lys(-Gly-Gly-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe from a few runs were pooled and subjected to hydrogenation to afford Boc-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe as a colorless film. δ_H (500 MHz; CD_3OD) 4.38-4.41 (2 H, m), 3.92-3.96 (4 H, m), 3.78-3.87 (2 H, m), 3.68 (3 H, d, *J* 11.7), 3.20 (2 H, s), 1.61-1.70 (2 H, m), 1.53 (2 H, d, *J* 0.7), 1.39-1.43 (11 H, m), 1.35-1.39 (5 H, m); LMRS (m/z) 631.3 (M+H) (Figure App2.10).



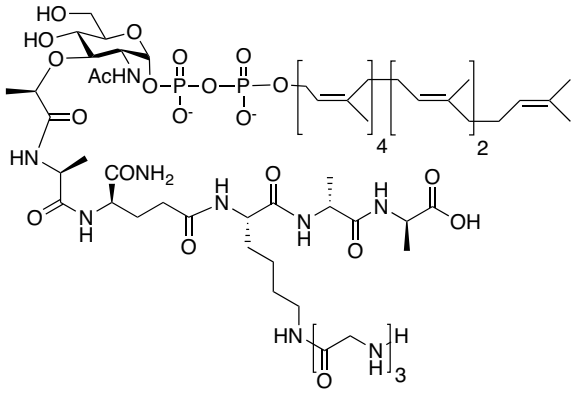
Boc-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (11). To a stirring solution that contains compound **10** (84 mg, 0.13 mmol) and TFA-Gly-OH (113 mg, 0.7 mmol) in DCM/DMF (3 mL/ 0.5 mL), was added HOBt-H_2O (30 mg, 0.2 mmol), DIEA (35 µL, 0.2 mmol) and EDC (38 mg, 0.2 mmol). The reaction mixture was concentrated and purified by HPLC. The product Boc-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe **(11)** was afforded as a colorless film (80 mg, 75%). δ_H (500 MHz; CD_3OD) 4.38-4.42 (1 H, m), 4.21 (1 H, dd, *J* 5.6 and 3.1), 4.03-4.07 (1 H, m), 3.88-3.97 (2 H, m), 3.81-3.83 (1 H, m), 3.68 (3 H, dd, *J* 20.6 and 15.2), 3.34-3.37 (1 H, m), 3.19-3.21 (1 H, m), 1.65-1.73 (1 H, m), 1.57-1.64 (1 H, m), 1.54 (1 H,

ddtd, *J* 9.9, 5.1, 2.1 and 0.9), 1.39-1.45 (4 H, m), 1.32-1.39 (4 H, m), 1.28-1.31 (2 H, m), 0.90-0.96 (2 H, m); LMRS (m/z) 806.1 ($M+Na^+$) (Figure App2.11).



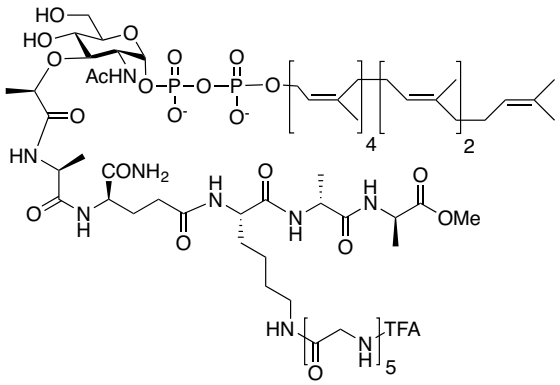
Gly-Lipid I (12). MurNAc-PP-heptaprenyl lipid (4 mg, 3 μmol) was evaporated twice with 0.5 M DIEA in MeOH (prepared by 274 μL DIEA in 3 mL MeOH) for ammonium salt exchange and dried under high vacuum for at least 1 h. While compound **3**, Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-TFA)-D-Ala-D-Ala-OMe (4 mg, 6 μmol) was stirred in 4 M HCl (3 mL) under N_2 for 20 min. The solution was concentrated to dryness and concentrated twice more from toluene to remove trace HCl, yielding HCl NH_2-L-Ala-D-*iso*Gln-L-Lys(-Gly-TFA)-D-Ala-D-Ala-OMe which was used without further purification. The protected peptide was dissolved in dry MeOH (500 μL) and was transferred to the vial containing dried MurNAc-PP-heptaprenyl residue under N_2. The mixture was treated with DIEA (0.5 M in MeOH, 10 μL, 9 μmol) followed by dimethoxytriazine-N-methylmorpholinium chloride (DMTMM, 2.5 mg, 9 μmol). After being stirred at room temperature for 5 h, the reaction was concentrated *in vacuo*, and purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% ACN/ 0.2% NH_4COO_3H. The 60% fraction contained the protected Gly-Lipid I product (0.7 mg, 7.3%). LRMS (m/z): 1565.7 (M-H), 781.4 $(M-2H)^{2-}$ (Figure App3.12). The protected compound (0.7 mg, 0.4 μmol) was dissolved in 100 μL 1:1 dioxane/H_2O and 20 μL 1 M LiOH in H_2O was added under stirring. Global protecting hydrolysis was observed after 30 min by ESI-

MA. The reaction was neutralized with NH_4Cl (100 µL), and then concentrated *in vacuo*. The residue was purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% ACN/ 0.2% NH_4COO_3H. The 50% fraction was concentrated *in vacuo* to give Gly-Lipid I (**12**) as a colorless film (0.3 mg). LRMS (m/z): 1454.7 (M-H), 726.9 $(M-2H)^{2-}$ (Figrue App2.13). HRMS (m/z): calcd for $C_{68}H_{115}N_9O_{21}P_2$ $(M-2H)^{2-}$ 726.8769, found 726.8769 (Figure App2.14).



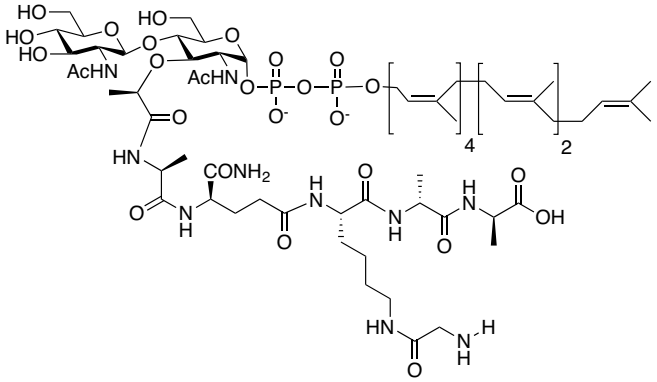
Gly_3-Lipid I (13). MurNAc-PP-heptaprenyl lipid (3.5 mg 3 µmol) was evaporated twice with 0.5 M DIEA in MeOH for ammonium salt exchange and dried under high vacuum for at least 1 h. While compound **5**, Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (5 mg, 6 µmol) was stirred in 4 M HCl (3 mL) under N_2 for 20 min. The solution was concentrated to dryness and concentrated twice more from toluene to remove trace HCl, yielding NH_2-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe which was used without further purification. The protected peptide was dissolved in dry MeOH (500 µL) and was transferred to the vial containing dried MurNAc-PP-heptaprenyl residue under N_2. The mixture was treated with DIEA (0.5 M in MeOH, 10 µL, 9 µmol) followed by dimethoxytriazine-N-methylmorpholinium chloride (DMTMM, 2.5 mg, 9 µmol). After being stirred at room

temperature for 4 h, the reaction was concentrated *in vacuo*, and purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% ACN/ 0.2% NH_4COO_3H. The 60% fraction contained the protected Gly_3-Lipid I product (1.8 mg, 18.8%), while the 10% fraction contained unreacted peptide material, and the 70% fraction contained unreacted MurNAc-PP-lipid. LRMS (m/z): 838.7 $(M-2H)^{2-}$ (Figure App3.15). The protected compound (1.8 mg, 1.1 μmol) was dissolved in 500 μL 1:1 dioxane/H_2O and 40 μL 1 M LiOH in H_2O was added under stirring. Global protecting hydrolysis was observed after 30 min by ESI-MS. The reaction was neutralized with NH_4Cl (200 μL), and then concentrated *in vacuo*. The residue was purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% ACN/ 0.2% NH_4COO_3H. The 50% fraction was concentrated *in vacuo* to give Gly_3-Lipid I as a colorless film (1.2 mg, 70 %) LRMS (m/z): 783.6 $(M-2H)^{2-}$ (Figure App2.16). HRMS (m/z): calcd for $C_{72}H_{121}N_{11}O_{23}P_2$ $(M-2H)^{2-}$ 783.8983, found 783.8987(Figure App2.17).



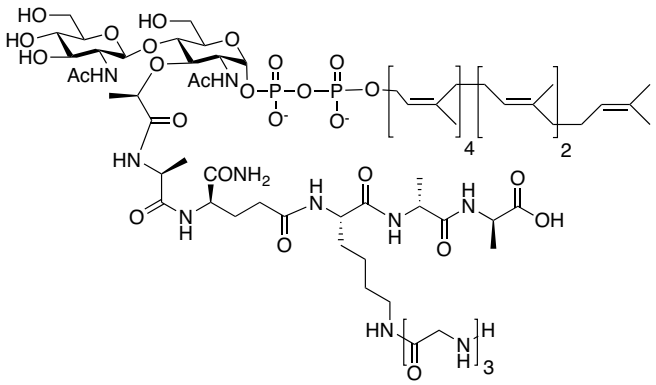
Protected Gly_5-Lipid I (14). MurNAc-PP-heptaprenyl (2.5 mg 3 μmol) was evaporated twice with 0.5 M DIEA in MeOH for ammonium salt exchange and dried under high vacuum for at least 1 h. While compound **7**, Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (5 mg, 5 μmol) was stirred in 4 M HCl (3 mL) under N_2 for 20 min. The solution

was concentrated to dryness and concentrated twice more from toluene to remove trace HCl, yielding NH_2-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe which was used without further purification. The protected peptide was dissolved in dry MeOH (500 µL) and was transferred to the vial containing dried MurNAc-PP-heptaprenyl residue under N_2. The mixture was treated with DIEA (0.5 M in MeOH, 10 µL, 9 µmol) followed by dimethoxytriazine-N-methylmorpholinium chloride (DMTMM, 2.5 mg, 9 µmol). The reaction progression was monitored over 5 h, but no product was observed on MS. The reaction was concentrated *in vacuo*, and purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% ACN/ 0.2% NH_4COO_3H. The 60% fraction was analyzed, and contained small amount of product. LRMS (m/z): 895.8 $(M-2H)^{2-}$ (Figure App2.18).



Gly-Lipid II (15). Gly-Lipid I was converted to Gly-Lipid II using a previously described enzymatic method.[10] A radiolabeled reaction was carried out in parallel. Briefly, to a tube containing Gly-Lipid I (75 µg, 50 nmol) dissolved in MeOH (24 µL), 50 mM HEPES buffer, 10 mM $MgCl_2$ in H_2O and incubated with 1 U/µL alkaline phosphatase and 0.5 mg/mL *E. coli* MurG in a final reaction volume of 160 µL for 1 h at room temperature. An additional aliquot of MurG (8 µL of 5 mg/mL stock) was added. After 1 h, the reactions were quenched with 160 µL

ice cold H_2O, and purified on a reverse phase C18 SPE column. After washing with 0.1% ammonium hydroxide, the desired compound was eluted with 0.1 % ammonium hydroxide in methanol. Fraction from non-radiolabeled experiments were analyzed by ESI-MS. Vacuum centrifugation afforded non-radiolabeled Gly-Lipid II; HRMS (m/z): calcd for $C_{76}H_{128}N_{10}O_{26}P_2$ $(M-2H)^{2-}$ 828.4165, found 828.4146 (Figure App2.19).



Gly_3-Lipid II (16). A similar approach was used to Gly_3-Lipid I was converted to Gly_3-Lipid II using MurG reaction as described above. HRMS (m/z): calcd for $C_{80}H_{134}N_{12}O_{28}P_2$ $(M-2H)^{2-}$ 885.4380, found 885.4347 (Figure App2.20).

3.6.2 Small-scale extraction of *S. aureus* Lipid II

The small-scale lipid extraction was modified from a previously published protocol.[17] An overnight culture (20 mL) of *S. aureus* RN4220 was diluted to an OD_{600} = 0.1, and allowed to grow to mid-exponential phase at 37 °C. The culture was divided into two partitions (10 mL each). One culture was treated with moenomycin (0.6 μg/mL) and the other was not. At the indicated time, 1 mL aliquot *S. aureus* taken out from each culture to read O.D. and then transferred to a glass tube containing 3.5 mL of a 2:1/$CHCl_3$: MeOH. The mixture was vortexed for 10 min at 25 °C, and any cell debris was removed by centrifugation for 10 min at 4,000 x g. The supernatant was collected and transferred to a new glass tube with 2 mL $CHCl_3$ and 1.5 mL

PBS. The mixture was vortexed for 10 min, and centrifuged for 10 min at 4,000x g to achieve phase separation. The bottom organic layer was collected and dried under N_2. The dried fractions were resuspended in 12 μL DMSO. Each fraction (2 μL) was analyzed by in Western blot analysis of BDL-labeled Lipid II (Figure 3.1).

3.6.3 Large-scale extraction of *S. aureus* Lipid II

Large-scale lipid extraction was performed on 6 L (4x 1.5L) of *S. aureus* RN4220 cultures. An overnight culture of *S. aureus* RN4220 in TSB media (15 mL) was used to inoculate each 1.5 L culture. The cultures were grown at 37 °C with shaking at 220 rpm. Moenomycin was added at a final concentration of 0.6 μg/mL to each 1.5 L culture when OD_{600} = 0.5-0.6 for accumulation of Lipid II. The moenomycin-treated cultures were grown for another 30 min before harvesting cell pellets. The pellets from 6 L (4 x 1.5 L) cultures were resuspended in 60 mL PBS (pH 7.4), and divided equally into 4 x 125 mL Erlenmeyer flasks, each of which contains a mixture of 52.5 mL 2: 1 MeOH: chloroform solvents. The mixture was stirred for 1 h at room temperature to ensure cell lysis. The mixture (about 70 mL) from each Erlenmeyer flask was poured into two Teflon tubes, which were spun down at 4000 x g for 10 min at 4 °C. The cell debris was visible as a pellet at the bottom of the tube, while the supernatant that contains the solubilized cellular contents were collected. The supernatants from two tubes were combined and poured into a 125 mL Erlenmeyer flask containing 30 mL chloroform and 22.5 mL PBS. The mixture was stirred at high speed for an hour for thorough mixing of the layers. The homogenized mixture was quickly poured into three clean Teflon tubes and centrifuged at 4000 x g for 10 min at 4 °C. It is important to quickly transfer the heterogeneous mixture into three tubes so that the composition of the mixture in each tube is roughly the same. In each Teflon

tube, a white interface layer was observed between the top aqueous and bottom organic layer. The top aqueous layer may appear hazy at first, but the haziness settles into the interface when the Teflon tube warms up to room temperature, giving a clear aqueous layer. The interface layer was collected by first removing the aqueous layer slowly using a Pasteur pipette. It is unavoidable to take up some volume of the aqueous layer while transferring the interface. The interface layer and the remaining organic layers are dried and analyzed by Western blot analysis of BDL-labeled Lipid II (Figure 3.2a).

For the second extraction to remove the Park nucleotide from the interface, the dried interface (from 4 x 1.5 L cell culture) was dissolved in 15 mL organic mixture of 1: 2 6M pyridinium acetate: n-butanol (prepared by mixing 51.5 mL glacial acetic acid with 48.5 mL pyridine), and washed with 15 mL of aqueous solvent (n-butanol saturated water) in a separation funnel. The aqueous layer was extracted again with 10 mL organic solvent (1: 2 6M pyridinium acetate: n-butanol) to maximize Lipid II extraction. The organic layers were combined and washed with aqueous solvent (n-butanol saturated water) (10 mL x 3) to remove the water-soluble Park nucleotide (Figure 3.2a). The clean organic layer was concentrated by rotary evaporation, and dissolved in MeOH for LC/MS quantification. For biochemical assays, the dried Lipid II was dissolved in DMSO, and was referred to as lipid extract.

3.6.4 Thin layer chromatography (TLC) analysis of phospholipids

Lipid extract dissolved in MeOH or phospholipids standards (CL: cardiolipin, PG: phosphotidylglycerol, LPG: lysyl-phosphotidylglycerol) in MeOH (at 5 mg/mL) were spotted on a TLC plate. The TLC was eluted in solvent $CHCl_3$: MeOH: CH_3COOH of 60: 30: 10, and detected by spraying with a solution of $CuSO_4$ in 8% phosphoric acid, and heating at 180 °C for

10 min until brown spots indicating phospholipids appeared (Figure 3.2b).

3.6.5 Western blot analysis of BDL-labeled Lipid II

The crude extract containing Lipid II was diluted in DMSO, and added to a mixture containing PBP4 (4 μM), BDL (3 mM) in a reaction buffer (12.5 mM HEPES (pH = 7.5), 2 mM $MnCl_2$, and 250 μM Tween-80) to reach a total volume of 10 μL with a final DMSO concentration of 20%. The reaction was incubated at room temperature for 1 h, and quenched with 10 μL of 2x SDS loading buffer. The final mixture (3 μL) was loaded onto a 4-20% gradient polyacrylamide gel and let run at 200 V for 40 min. The products were transferred to Immun-Blot PVDF membrane (BioRad) at 25 V (fixed) 1.3 A (variable) for 15 min. BDL-Lipid II was detected by blotting with streptavidin-HRP (1:10000 dilution), visualized using ECL Prime Western Blotting Detection Reagent (GE Ambersham) on Azure C400 imaging system (Figure 3.2b).

3.6.6 LC/MS analysis of delipidated *S. aureus* Lipid II

A small amount of the interface lipid extract in DMSO (10 μL) was incubated with H_2O (80 μL) and 100 mM ammonium acetate at pH 4.2 (10 μL). The mixture was heated at 100 °C in a heating block for 30 min. Under these conditions, the Lipid II is hydrolyzed at the phosphodiester linkage. The mixture was lyophilized, resuspended in 20 μL H_2O, and subjected to LC/MS analysis, conducted with ESI-MS operating in negative mode. The instrument was equipped with a Waters Symmetry Shield RP18 column (5 μM, 3.9 x 150 mm) with a matching column guard. The fragments were separated using the following method: 0.5 mL/min H_2O (0.1% formic acid) for 5 min followed by a gradient of 0 % acetonitrile (ACN) (0.1% formic

acid)/H_2O (0.1% formic acid) to 20% ACN (0.1% formic acid)/H_2O (0.1% formic acid) over 40 min. The molecular ion corresponding to expected disaccharide muropeptide monophosphate fragment was extracted from chromatograms (Figure 3.3). MS/MS chromatograms of delipidated *S. aureus* Lipid II were shown in Figure App2.21.

3.6.7 Quantifications of delipidated *S. aureus* Lipid II

The interface lipid extract in DMSO (4 μL) was mixed with varying concentrations of synthetic Lys-Lipid II in DMSO (4 μL of 200 μM, 100 μM or 50 μM) respectively, which was then subjected to heat degradation in buffer (10 mM ammonium acetate, pH 4.2) at 100 °C for 30 min. The mixture was lyophilized, redissolved in 20 μL H_2O, and subjected to LC/MS analysis, conducted with ESI-MS operating in negative mode. The LC/MS run method was indicated above. The molecular ions corresponding to both degraded fragments (from synthetic Lys-Lipid II and native *S. aureus* Lipid II) were extracted from chromatograms. The amount of native Lipid II was estimated using a standard curve of the synthetic Lys-Lipid II (Figure App2.22).

An alternative quantification method was performed by biotinylation of Lipid II. In this method, the interface extract in DMSO at various dilutions were labeled with BDL using *S. aureus* PBP4, and the BDL-labeled Lipid II was then subjected to Western blot detection, as previously discussed. Densitometry analysis of the BDL-Lipid II (from the interface extract) was done using BDL-labeled Lys-Lipid II (Lys-Lipid II of known concentrations were labeled with BDL using *S. aureus* PBP4) as a standard curve on ImageJ (Figure App2.22).

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Chapter Four: Reconstitution of peptidoglycan assembly by *Staphylococcus aureus* PBP2

4.1 Introduction

This chapter describes the reconstitution of the transpeptidase activity of *S. aureus* PBP2, the only essential Class A PBP in *S. aureus*.[a] Access to useful quantities of native *S. aureus* Lipid II, as discussed in Chapter Two and Three, has enabled the development of *in vitro* TP assays. This allows us to characterize beta-lactam antibiotics by directly monitoring their inhibition of TP activity *in vitro* for the first time. The work in this chapter lays the groundwork for future discovery of novel classes of TP inhibitors such as non-covalent inhibitors.

4.1.1 PBP2 is the essential Class A PBP in *S. aureus*

S. aureus PBP2 is an essential bifunctional PBP (Class A) that has both a peptidoglycan glycosyltransferase (PGT) and a transpeptidase (TP) domain. The PGT domain couples Lipid II monomers to make glycan strands, while the TP domain crosslinks adjacent strands via a short peptide. The identity of PG crosslinks is distinct in different bacteria; in *S. aureus*, the peptide cross-link is a pentaglycine bridge (Figure 4.1).[1] The cellular importance of the PGT and TP domains of *S. aureus* PBP2 has been probed by genetic inactivation of each active site residue.[2,3] Its TP domain was found essential for viability in methicillin-sensitive *S. aureus* (MSSA), and can only be replaced by PBP2a in methicillin-resistant *S. aureus* (MRSA) strains.[2] However, the PGT domain of PBP2 is required for the full expression of methicillin resistance in MRSA, suggesting a cooperation between the PBP2 PGT activity and PBP2a TP activity.[3] In addition, the PBP2 TP domain has been suggested to cooperate with PBP4 to produce highly cross-linked PG in *S. aureus*.[4]

[a] Class A PBP refers to bifunctional PBP, which has both PGT and TP domains.

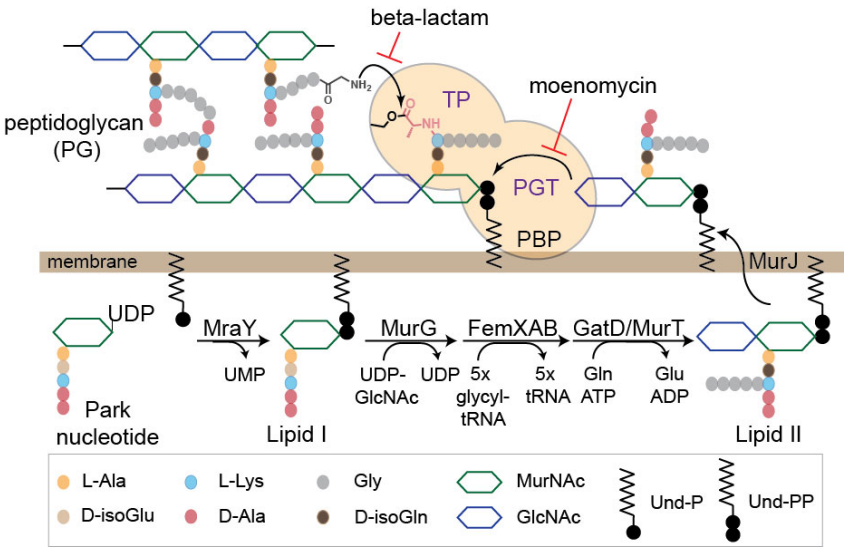

Figure 4.1. Schematic depiction of *S. aureus* PG biosynthetic pathway. *S. aureus* Lipid II contains a unique peptide. The PGT and TP domains of penicillin-binding proteins (PBPs) accomplish the final stages of PG assembly and are good antibiotic targets. PBP2 is the major PBP in *S. aureus*.

The PGT activity of *S. aureus* PBP2 was characterized *in vitro* by Barrett *et al.* (2005) using synthetic Lys-Lipid II substrate, which was the first reconstitution of a Gram-positive Class A HMW PBP.[5] In this work, a full-length construct of *S. aureus* PBP2 was heterologously overexpressed and purified. This construct exhibited optimal activity at pH 4.5-5.5, while the activity decreased in the presence of any detergent and did not depend on the presence metal ions. Under such optimal condition, *S. aureus* PBP2 PGT had a catalytic efficiency (k_{cat}/K_m: 2.4 x 10^3 $M^{-1}s^{-1}$) comparable to that of the well-characterized *E. coli* PBP1b (k_{cat}/K_m: 3.5 x 10^4 $M^{-1}s^{-1}$).[6] However, it was proposed that the native pentaglycine-containing Lipid II may enhance the catalytic efficiency of *S. aureus* PBP2. Wang *et al.* (2008) showed that purified PGTs from different organisms could produce PG polymers of variable lengths. The full-length *S. aureus* PBP2 produces glycan polymers of about 15 disaccharide units *in vitro*.[7] PG polymers of proper lengths in *S. aureus* are thought to be important for the placement of the division plane to ensure

orthogonal divisions.[8] A moenomycin-resistant *S. aureus* mutant, which contains a point mutation in the PGT domain of PBP2 protein (PBP2 Y196D), manifests aberrant cell divisions.[8] Compared to wildtype PBP2 protein, PBP2 Y196D mutant protein displayed a distributive mechanism *in vitro* where short PG polymers were released after each coupling.[8] Several crystal structures of different bacterial PGTs in complex with moenomycin have been reported, in which moneomycin binds in the extended PGT active site.[9-11] The Y196D mutation in PBP2 resides in the distal region of the PGT active site cleft, which is proximal to the A, B rings of moenomycin. The fact that a point mutation in the PGT domain of *S. aureus* PBP2 confers a 25-fold increase in the MICs of moenomycin (0.5 μg/mL to 12.5 μg/mL) establishes that PBP2 is the lethal target of moenomycin.[8]

While much is known about *S. aureus* PBP2 PGT activity, its TP activity remains to be demonstrated. In the crystal structure of *S. aureus* PBP2, the C-terminal TP domain is separated from the N-terminal PGT domain by a small linker region.[11] The active site of the TP domain contains the characteristic motifs (SXXK, SXX, KTG) with S398 (underlined) being the active site residue.[12] Several beta-lactam resistant *S. aureus* strains that bear mutations in the PBP2 TP domain have been isolated clinically or in laboratory conditions.[4,13] However, despite its importance as the target of beta-lactams, *S. aureus* PBP2 TP activity has not been characterized due to the lack of an appropriate substrate. Notably, the Ac_2-L-Lys-D-Ala-D-Ala tripeptide substrate, which has been used for probing LMW PBPs, is not suitable for studying the TP activities of HMW PBPs, since the TP domain and the PGT domain are coupled in HMW PBPs.[14] The TP activities of *E. coli* HMW PBPs (PBP1a and PBP1b) have been shown to require ongoing PGT activities.[14,15]

In this chapter, we used native *S. aureus* Lipid II substrate to characterize PBP2 activity *in vitro*. The composition of PG assembled by PBP2 *in vitro* greatly resembles that of the isolated sacculus from *S. aureus*. This observation is in line with the fact that PBP2 is the major TP in *S. aureus*. In this work, we have developed a facile activity assay that directly reports on the TP-mediated cross-linking and D-amino acid exchange reaction, enabling studies of beta-lactam inhibition of transpeptidation.

4.1.2 Traditional competition binding assays to study beta-lactams

In the absence of a transpeptidase activity assay, competition binding assays have been the prevalent approach to study beta-lactam interaction with bacterial PBPs, in which the concentration of a beta-lactam of interest needed to prevent binding of a labeled penicillin is assessed.[16] Different beta-lactams display greatly varied affinities to PBPs in a given organism. For instance, mecillinam, a broad-spectrum beta-lactam, demonstrates specific binding to *E. coli* PBP2, an essential enzyme involved in cell elongation of the rod-shaped *E. coli*.[17] *S. aureus* PBP3, which has a high homology to *E. coli* PBP2, also demonstrates high affinity for mecillinam.[18]. Consistent with the fact of *S. aureus* PBP3 is non-essential, mecillinam exhibits poor antibacterial activity against *S. aureus*. Although competition binding assays have allowed one to delineate the possible target(s) for a beta-lactam drug in bacteria, such assays are not desirable for studying non-covalent PBP inhibitors or beta-lactams with a fast rate of release from the enzyme, since a large excess of penicillin is necessary for labeling in the experiment and may result in an underestimation of the inhibitor's binding affinity. For instance, the cefaclor-bound PBP2 species has a high deacylation rate at 37 °C, despite cefaclor having a high affinity for the enzyme.[19] If the incubation time exceeds the half-life of the covalent adduct

(penicilloyl-PBP), *S. aureus* PBP2 can be labeled by the excess penicillin to result in a misleading readout.[20] Therefore, a direct activity assay to study beta-lactam inhibition of PBPs is needed.

Using the TP assays developed in this chapter, we characterized the inhibitory activities of a panel of beta-lactams on *S. aureus* PBP2. We showed that the TP activity assay is useful for studying inhibitors that show reversible binding, such as cefaclor. We also investigated the TP activities of PBP2 mutant proteins that are implicated antibiotic resistance and confirmed their resistance to be associated with specific beta-lactams. Furthermore, we analyzed the substrate specificity of *S. aureus* PBP2 and revealed that it required a certain length of glycine branch for transpeptidation *in vitro*. These results provide a platform for studying the activities of other bacterial PBPs and for the continuing search of new classes of TP inhibitors.

4.2 Purification of *S. aureus* PBP2

Like other HMW PBPs, *S. aureus* PBP2 is a transmembrane (TM) protein with an N-terminal TM helix that anchors it to the cytoplasmic membrane. We have previously cloned, overexpressed and purified a full-length construct of *S. aureus* PBP2, and reconstituted its PGT activity using synthetic Lys-Lipid II.[5] However, an anionic detergent, sodium lauryoyl sarcosinate, was necessary to solubilize the full-length PBP2 from the membrane fraction during purification. The purified full-length protein appeared aggregated on size-exclusion chromatography (SEC) analysis. As a result, the observed activity may come from only a small percentage of the well-folded protein in the purification preparation. To obtain a soluble *S. aureus* PBP2 construct, we cloned a PBP2 [M59-S716] construct with its N-terminal TM helix

truncated.[b] To facilitate solubilization of *S. aureus* PBP2 protein, we added 40 mM of CHAPS detergent (CMC of CHAPS is about 8 mM) to the purification buffer. A significant amount PBP2 [M59-S716] was solubilized under this condition. During the Ni-NTA column chromatography step, we exchanged the detergent to 0.28 mM LDAO (CMC of LDAO is about 1 mM), a common zwitterionic detergent for membrane protein purification and crystallization.[21] The PBP2 [M59-S716] appeared well-folded in this storage buffer at pH 8.0 based on SEC analysis. From 1 L of *E. coli* culture for protein expression, 5-10 mg of *S. aureus* PBP2 [M59-S716] was obtained after Ni-NTA and SEC purifications. This was a major improvement from the previous purification of the full-length construct, where only 1-2 mg PBP2 protein (that was aggregated) was obtained per liter of culture.[5] Since the protein demonstrated desirable purity (Figure 4.2), we subjected it to biochemical studies without additional steps of purification.

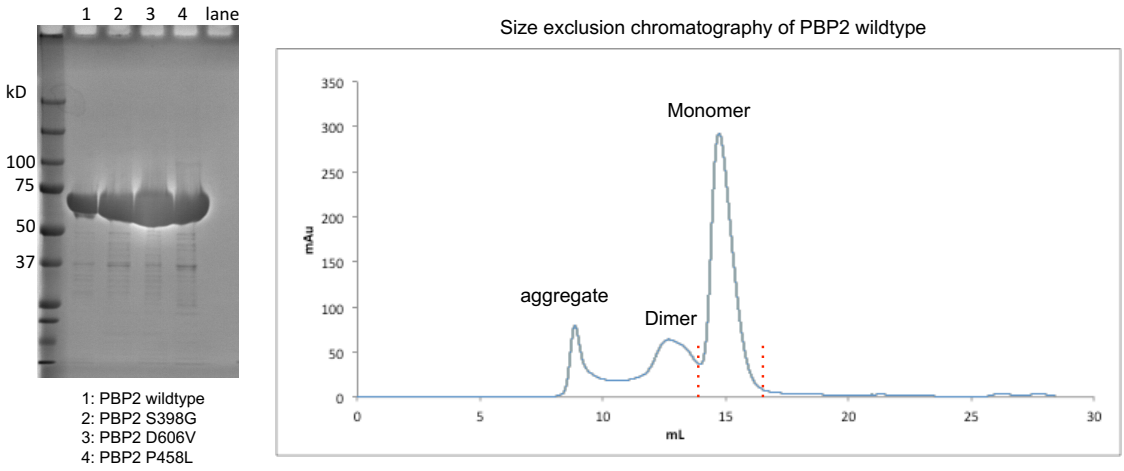

Figure 4.2. Purification of *S. aureus* PBP2 [59-716] construct and mutants. Coomassie gel showing PBP2 was purified in homogeneity (left), size-exclusion chromatography (SEC) trace of *S. aureus* [59-716] wildtype. A small aggregated peak was eluted in the void volume, a small peak that represents the dimer and the predominant monomeric peak. The red dotted line indicated the fractions that were collected.

[b] This work was done in collaboration with a graduate student, Jeep Veerasak Srisuknimit and two undergraduate students, Alan Yang and Akeem Pinnock. The purification protocol was modified from Lovering *et al.* (2007).

4.3 Reconstitution of the PGT and TP activities of *S. aureus* PBP2

To reconstitute the activities of *S. aureus* PBP2, we used native Lipid II substrate prepared by direct isolation from *S. aureus* (see Chapter Three). As mentioned earlier, the PGT activity of *S. aureus* PBP2 has been studied using model Lys-Lipid II substrate,[5] but characterization of its TP activity has not been possible due to a lack of the appropriate Lipid II substrate. The presence of the pentaglycine branch on native Lipid II allows us to study *S. aureus* PBP2 TP activity for the first time. We adopted the LC/MS assay that was used to study PBP4 activity as described in Chapter two for investigating PBP2 PGT and TP activities.[22] The purified *S. aureus* PBP2 protein was incubated with native Lipid II substrate at room temperature. After one hour of incubation, the product was digested with mutanolysin to give the disaccharide muropeptides, which were further reduced by $NaBH_4$ to prevent anomerization of the reducing end on MurNAc.[c] The mixture was then subjected to LC/MS analysis (Figure 4.3a). We identified monomeric muropeptide (**A**), as well as dimeric (**B**) and trimeric (**C**) muropeptides. The presence of peak **A** indicated the PGT activity of *S. aureus* PBP2, while the presence of peak **B** and **C** suggested successful TP activity of PBP2. Incubation of *S. aureus* PBP2 TP active site mutant with Lipid II resulted in only monomeric muropeptide (**A**), identical to what was observed when the monofunctional PGT SgtB was used for Lipid II polymerization (Figure App3.1). The composition of muropeptides from PG produced by PBP2 *in vitro* closely resembles that produced by digestion of the isolated *S. aureus* sacculus (Figure 4.3b). Hence, using native Lipid II substrate, we have reconstituted both glycan polymerization and strand cross-linking by the essential enzyme *S. aureus* PBP2. It should be mentioned that acidic buffer condition (pH 5.5) that was used previously for the full-length PBP2 construct was not optimal

[c] Reduction of the anomeric carbon on MurNAc increases its ionization on mass spectrometry.

for our studies here, since the truncated PBP2 demonstrated better *in vitro* activity at neutral pH and the acidic buffer interfered with the LC/MS analysis.

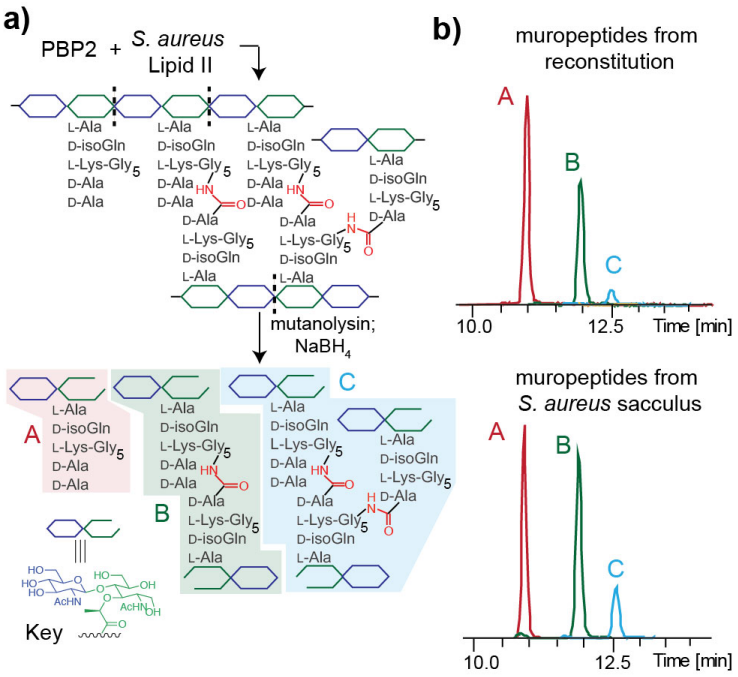

Figure 4.3. *S. aureus* PBP2 uses native Lipid II to make cross-linked PG *in vitro*. (a) Schematic of PBP2 reaction to produce PG and muropeptide fragments resulting from degradation. (b) LC/MS EICs of the degraded products from PBP2 reaction (top) and from degraded sacculus (bottom). Peak **A**, monomeric muropeptide; peaks **B** and **C**, cross-linked dimeric and trimeric muropeptides, respectively. The following ions were extracted: A: 1254.5856 (m+H); B: 1029.0617 ($[m+2H]^{2+}$); and C: 1194.2204 ($[m+3H]^{3+}$). (See materials and methods for detailed protocols).

We next sought to develop a faster assay to monitor PBP2 activity. The LC/MS analysis of transpeptidation relies on a high-resolution mass spectrometer to detect the cross-linked products, and the throughput is slow (i.e. each LC/MS run takes 50 min). We examined whether *S. aureus* PBP2 can incorporate exogenous D-amino acids during *in vitro* PG assembly. If so, D-amino acid incorporation can provide a facile assay to study *S. aureus* PBP2 TP activity *in vitro*. It is known that several HMW PBPs in *E. coli* and *B. subtilis* are capable of incorporating a variety of D-amino acids into PG *in vitro*.[23,24] We recently demonstrated that the LMW *S. aureus* PBP4 can incorporate a variety of functionalized D-amino acid derivatives into either pre-formed

PG or monomeric Lipid II *in vitro*; in particular, the incorporation of biotinylated-D-lysine (BDL) has led to a simple and sensitive assay to detect the cellular pools of Lipid II in bacteria.[22] Therefore, we chose BDL as a probe to investigate *S. aureus* PBP2 TP activity *in vitro*. Here, the purified PBP2 protein was incubated with native Lipid II and BDL at room temperature for one hour, and then the reaction was quenched and loaded onto an SDS-PAGE gel for separation, followed by Western blot analysis using a streptavidin-HRP conjugate. Surprisingly, the only signal detected was found at a location that corresponded to the loading well of the gel (Figure 4.4a, left lane), suggesting that the product was too highly cross-linked to enter the SDS-PAGE gel matrix.[25,26] To resolve the cross-linked product, we added lysostaphin to the reaction mixture prior to analysis by gel electrophoresis. Lysostaphin is an endopeptidase that specifically cleaves cross-linked pentaglycine bridges in *S. aureus* PG.[27] In the lysostaphin-treated sample, we detected strong signals across a wide range of molecular weights on the blot (Figure 4.4a, right lane). When the reaction sequence was carried out using PBP2 TP active site mutant, no signal was observed in the presence or absence of lysostaphin, indicating that BDL labeling was specific to PBP2 TP activity (Figure App3.2). Therefore, we conclude that PG polymers produced by the PGT domain of *S. aureus* PBP2 undergo TP-mediated cross-linking as well as BDL incorporation, resulting in a cross-linked macromolecule labeled with BDL.

As *S. aureus* PBP2 mediate PG cross-linking and D-amino acid incorporation (into PG) via the same acyl-enzyme intermediate (i.e. the activation step is identical), the two reactions are competing reactions (Figure 1.6). Indeed, we observed that as the concentration of Lipid II increases from 5 μM to 20 μM in the reaction, while keeping the concentrations of PBP2 (1 μM) and BDL (3 mM) constant, the signal of BDL-labled PG became fainter (data not shown). This implied that PG cross-linking was favored over BDL incorporation by *S. aureus* PBP2 when

higher concentration of Lipid II substrate was used in the reaction. Although the concentration of exogenous BDL used was orders of magnitude higher than that of the Lipid II substrate, we assume that the close proximity of the pentaglycine branch in PG to the donor peptide may dramatically enhance the effective concentration for cross-linking as the Lipid II substrate concentration increases in the reaction mixture. However, at a lower concentration of Lipid II substrate, cross-linking and BDL labeling of PG by PBP2 can both occur readily; lysostaphin treatment allows cleavage of PG cross-links to yield linear PG polymers labeled with BDL for the ease of analysis. In summary, BDL labeling of PG by *S. aureus* PBP2 provides a facile and qualitative readout for PBP2 transpeptidation reaction.

4.4 Assessing the inhibitory effects of beta-lactams

To demonstrate the utility of this activity assay, we investigated the inhibitory effects of a panel of beta-lactams. As mentioned earlier, studies of beta-lactams have relied on competition binding assays in which a radiolabeled or fluorescent penicillin is used as a probe.[16,28,29] Beta-lactam inhibition of the TP enzymatic activity of PBPs, however, has not been directly demonstrated. Here, we briefly incubated *S. aureus* PBP2 with an increasing concentration of different beta-lactams prior to running the reactions (Figure 4.4 and Figure App3.2). Cefalor, a second-generation cephalosporin, has been considered to be a weak binder of *S. aureus* PBP2 in the competition binding assay.[20] It was subsequently determined that cefaclor actually has a high affinity for PBP2 but undergoes rapid deacylation,[19] resulting in a misleading readout by this. The half-life of cefaclor acyl-PBP2 was approximately 10 min at 37 °C.[19] Therefore, the incubation time and the temperature of the reaction are important variables in the binding assay. If the time of radiolabeling exceeds the half-time of the acyl-enzyme intermediate, the binding of

the antibiotic to PBP will be significantly underestimated. In our activity assay, we found that cefaclor potently inhibited PBP2 activity at a concentration 5x that of the enzyme (5 μM of cefaclor versus 1 μM of PBP2). We did not observe significant differences in cefaclor inhibitory activities with either shorter (10 min) or longer (60 min) incubation time, but the amount of cefaclor required to inhibit *S. aureus* PBP2 activity *in vitro* varies with the concentration of PBP2 used in the reaction. In addition, mecillinam, an extended spectrum beta-lactam, which demonstrates poor antibacterial activity against *S. arueus,* has been reported to have poor affinity for *S. aureus* PBP2.[20] Indeed in our assay, it showed no detectable inhibition of PBP2 activity up to 20-fold excess of PBP2 concentration. Other beta-lactams including penicillin G, methicillin, and imipenem also demonstrate potent inhibitory activities in line with their binding data (Figure App3.2).[20,30,31] The direct *in vitro* activity assay allows us to evaluate the inhibitory activities of beta-lactams in a purified system conviently.

We next examined whether the TP activity assay would be useful for assessing the activities of resistance mutations upon beta-lactam inhibition. Several laboratory and clinical isolates of *S. aureus* strains that display resistance to certain beta-lactams have been identified, and the mutations are mapped to the *pbp2* gene.[4,13] In the case of a ceftizoxime-resistant *S. aureus* strain, a PBP2 P458L substitution has been shown to be responsible for resistance. The P458L mutation occurs in the TP cleft of *S. aureus* PBP2 (which is close to the TP active site residue). The mutant protein demonstrates a lower binding affinity for ceftizoxime than the wildtype enzyme.[4] However, it is not known if the P458 residue in PBP2 specifically interacts with ceftizoxime, due to a lack of the crystal structure of *S. aureus* PBP2 in complex with ceftizoxime. Moreover, the TP activity of PBP2 P458L mutant has not been characterized previously. We overexpressed and purified the *S. aureus* PBP2 P458L mutant protein, and

analyzed its activity. We found that the TP domain of PBP2 P458L variant active and its tolerance to ceftizoxime was about ten fold higher than the wildtype enzyme (Figure 4.4d). The mutant enzyme remained susceptible to oxacillin inhibition, consistent with the observation that the strain containing this mutation was more susceptible to oxacillin killing (Figure App3.2).[4] In contrast, a PBP2 D606V variant, found in several borderline oxacillin resistant *S. aureus* isolates,[13] tolerated more oxacillin than the wildtype enzyme did in the activity assay (Figure App3.2). Our observation establishes the mechanistic basis of the observed resistance.

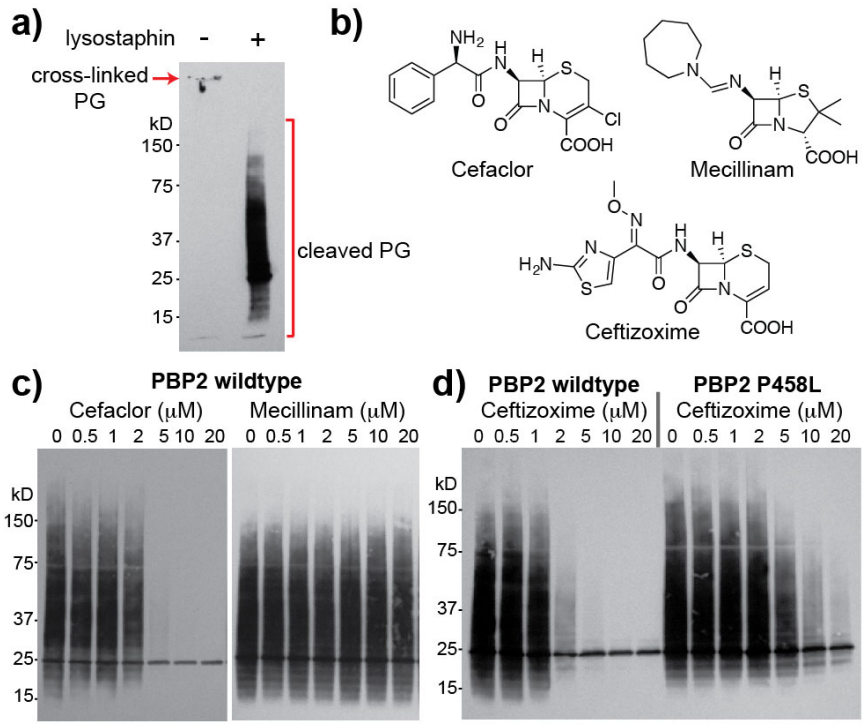

Figure 4.4. Beta-lactams inhibit *S. aureus* PBP2 TP activity. (a) Lysostaphin allows entrance of the BDL-labeled PG into the gel. (b) Structures of beta-lactams examined in c-d. (c) Cefaclor has potent inhibitory activity against PBP2, while mecillinam does not. The signal at 25 kD derives from non-specific binding of the conjugate to lysostaphin. (d) PBP2 resistant mutant P458L TP variant shows TP activity in the presence of higher doses of ceftizoxime. (See materials and methods for detailed protocols).

Given bacterial TP is an important antibiotic target, it is surprising that beta-lactams represent the only class of TP inhibitors in the clinic. Discovery of non-covalent TP inhibitors

has not been possible with the current competition binding assays. We showed that our activity assay is compatible with inhibitors that have a fast rate of release from the enzyme, such as cefaclor.[19] Therefore, we expect the assay to greatly facilitate the discovery of novel non-covalent TP inhibitors.

4.5 Investigating the substrate specificity of *S. aureus* PBP2

We also analyzed the substrate specificity of *S. aureus* PBP2 with Gly- and Gly_3-Lipid II variants that were isolated from the *S. aureus* Δ*femA* and Δ*femB* mutant respectively (see Chapter Three). As introduced in Chapter One, FemA and FemB are enzymes involved in the synthesis of the pentaglycine branch in Lipid II in *S. aureus*, where FemA attaches the second and third glycines, FemB attaches the last two glycines.[32] Analysis of PG structures in Δ*femA* and Δ*femB* mutants revealed a dramatic reduction in the level of PG cross-linking, with monoglycine bridges in the Δ*femA* mutant and triglycine bridges in the Δ*femB* mutant.[33] This observation suggests that one or more PBPs in *S. arueus* must be able to cross-link the shorter glycine branches in both mutants. Using similar reaction conditions to those described in Chapter 4.3, we incubated Gly- or Gly_3-Lipid II with purified *S. aureus* PBP2 protein, followed by mutanolysin/$NaBH_4$ treatment and LC/MS analysis (Figure 4.5). With Gly-Lipid II, the un-cross-linked Gly muropeptide peak ($A_{x=1}$) was observed predominantly, while a small peak corresponding to the dimer product was found. However, with Gly_3-Lipid II, both monomeric ($A_{x=3}$) and dimeric muropeptides ($B_{x=3}$) were present. This indicates that *S. aureus* PBP2 can use triglycine branch as a nucleophile for cross-linking, but not the monoglycine branch under the *in vitro* experimental condition. Whether *S. aureus* PBP2 is responsible for PG cross-linking in Δ*femA* and Δ*femB* mutants *in vivo* remains to be answered. In addition, we observed *S. aureus*

PBP2 could incorporate D-amino acids during PG assembly with Gly- or Gly_3-Lipid II substrate. Taken together, *S. aureus* PBP2 demonstrates stringent substrate requirement for acceptors in cross-linking, where Gly-Lipid II substrate cannot be used efficiently *in vitro*. Nevertheless, the identity of the glycine branch on Lipid II does not affect its suitability as a donor substrate for *S. aureus* PBP2 TP activity.

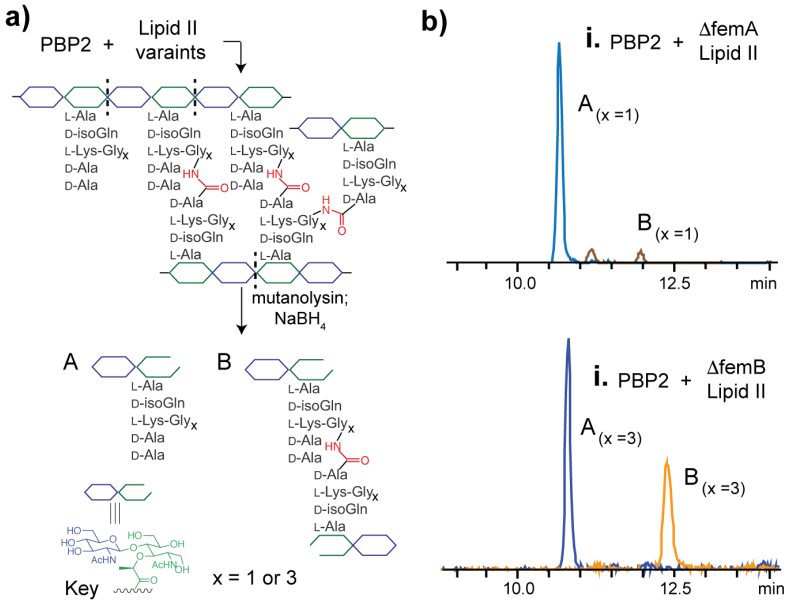

Figure 4.5. *S. aureus* PBP2 demonstrates substrate specificity in transpeptidation. (a) Schematic of PBP2 reaction to produce PG and muropeptide fragments resulting from degradation. (b) LC/MS EICs of the degraded products from PBP2 reaction with Gly-Lipid II (top) and with Gly_3-Lipid II (bottom). Peak **A**, monomeric muropeptide; peak **B**, cross-linked dimeric muropeptide. The following ions were extracted: A(x=1): 1205.4997 (m+H); B(x=1): 1960.9445 ($[m+H]^+$); and A(x=3): 1139.5426 (m+H); B(x=3): 1095.0188 ($[m+2H]^{2+}$). (See methods and materials for detailed protocols).

4.6 Conclusion and future studies

In summary, we have reconstituted the TP activity of *S. aureus* PBP2 for the first time using native Lipid II substrate and developed a facile activity assay to study beta-lactam inhibition *in vitro*. The availability of a TP activity assay opens up the possibility to screen for novel classes of PBP inhibitors. TPs are important antibiotic targets; however, beta-lactams represent the only class of antibiotics that target these enzymes. Although there were several

attempts to identify new classes of TP inhibitors using computational docking methods recently,[34] a systematic approach to discover potential TP inhibitors is lacking. We are currently working towards adopting our Western blot-based TP activity assay into a high-throughput screening assay to enable systematic discovery of new classes of PBP inhibitors. The fact that practical quantities of native Lipid II substrate can be readily accumulated and isolated from bacteria makes its cost-effective to develop a high-throughput screening assay.

In our efforts to reconstitute *S. aureus* PBP2 activity, we have established a reliable and generalizable route to obtain native Lipid II substrate (see Chapter Three), which provided important foundations for future *in vitro* studies of other bacterial TPs. We are currently continuing the investigation of the substrate specificity of *S. aureus* PBPs. If *S. aureus* PBP2 has an intrinsic preference for Gly_5- and Gly_3- over Gly-Lipid II, there must be another *S. aureus* PBP that can cross-link the Gly-Lipid II, since the *S. aureus* Δ*femA* mutant is still viable and its PG contains monoglycine cross-bridges. Moreover, the TP activities of Class B HMW PBPs, such as *S. aureus* PBP1, PBP2a and PBP3, are not well understood. Banzhaf *et al.* (2012) showed that the presence of *E. coli* PBP2 (a Class B HMW PBP) stimulates the TP activity of *E. coli* PBP1a (a Class A HMW PBP), as measured by the attachment of *in vitro* radiolabeled PG to sacculi.[35] However, *E. coli* PBP2 did not demonstrate any TP activity alone or with a PBP1a TP mutant. Therefore, characterization of Class B HMW PBPs still awaits, and understanding their activities may enable better strategies to target them.

4.7 Materials and Methods

4.7.1 General Methods

S. aureus strain was grown at 37 °C in Tryptic Soy Broth (TSB) media under aeration with shaking. Native *S. aureus* Lipid II was obtained by direct isolation from *S. aureus* culture as described in Chapter Three. LC/MS chromatograms were obtained on an Agilent Technologies 1100 series LC-MSD instrument using electrospray ionization (ESI). HRMS data was obtained on a Bruker Maxis Impact LC-q-TOF Mass Spectrometer using ESI. Western blots were developed using Biomax Light Film (Kodak) or imaged using Azure C400 imaging systems. ImageJ was used to quantify blots.

4.7.2 Cloning of *S. aureus* PBP2[M59-S716] and mutants

The *S. aureus pbp*2[M59-S716] construct was cloned into pET42a(+) plasmid using Gibson assembly protocol. Briefly, the nucleotide region of *S. aureus pbp2* gene encoding amino acid 59 to 716 was amplified using the following primer pair:
5'- TTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAGCACCTGCTTTTACCGA-3' and
5'- TGGTGGTGGTGGTGGTGCTCGAGAGATTGTTGAGATCTAGTATTGTTATTTGAT -3', where the underlined regions indicate sequences annealing to the PBP2 gene, and the W59 was mutated to become the new N-terminal methionine. The PCR product was analyzed and purified using agarose gel electrophoresis. The pET42a(+) plasmid was double-digested using restriction endonucleases NdeI and XhoI in the CutSmart buffer, followed by heat inactivation of the restriction endonucleases. The purified PCR product and the linearized vector were assembled using Gibson assembly protocol. The inserted *pbp2*[M59-S716] gene was confirmed by sequencing (Beckman Coulter Sequencing Facility). *E. coli* NovaBlue strain was used for

cloning. Point mutants of *pbp*2[M59-S716] were made with the appropriate in the parent plasmid using QuickChange site-directed mutagenesis kit (Stratagene). The constructs were confirmed by sequencing.

*pbp*2[M59-S716] S398G mutant was made using the following primer pair:

5'-CAACAGATCCTCACCCTACTGGTGGATCTTTAAAACCTTTCTTAGCGTAT-3' and

5'-ATACGCTAAGAAAGGTTTTAAAGATCCACCAGTAGGGTGAGGATCTGTTG-3'

*pbp*2[M59-S716] P458L mutant was made using the following primer pair:

5'-GACAAAGTTTCAATATCCTAGCTTTAAAAG-3' and

5'-CTTTTAAAGCTAGGATATTGAAACTTTGTC-3'

*pbp*2[M59-S716] mutant was made using the following primer pair:

5'-AATGCAGCGAAAGTCGTGTGG-3' and

5'-CCACACGACTTTCGCTGCATT-3' (Mutagenized codon is underlined).

4.7.3 Overexpression and purification of *S. aureus* PBP2 and mutants.

The plasmid encoding *S. aureus* PBP2[M59-S716] collected from the NovaBlue cloning strain was transformed into *E. coli* BL21(DE3) culture for overexpression and purification. A 15 mL culture of *E. coli* BL21 (DE3) harboring the plasmid encoding for *S. aureus* PBP2[M59-S716] grown in LB supplemented with 50 μg/ mL kanamycin was grown overnight, which was then used to inoculate 1.5 L LB medium (1:100) supplemented with 50 μg/mL kanamycin. The culture was grown at 37 °C with shaking until OD_{600} reached 0.4-0.5, and then cooled down to 17 °C before induction with 0.5 mM IPTG for 17 h with shaking. Cells were harvested by centrifugation (5250 x g, 20 min, 4 °C) and pellets were resuspended on ice with 30 mL of lysis buffer (10 mM Tris pH 8.0, 1M NaCl, 10 mM $MgCl_2$, 10% v/v glycerol and 40 mM CHAPs) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 μg/mL DNase

inhibitor. The resuspended cells were passed through a cell disrupter (3 x 10,000 psi, 4 °C) for three times. The cell lysate was then pelleted by ultracentrifugation (100,000 x g, 30 min, 4 °C). The resulting supernatant containing PBP2[M59-S716] protein was applied to 1.5 mL pre-washed Ni-NTA resin (Qiagen) at 4 °C (the Ni-NTA resin was washed with dH_2O and equilibrated with lysis buffer). After collecting flow through (FT), the resin was washed with buffer A (10 mM Tris pH 8.0, 0.2 M NaCl, and 0.28 mM LDAO) to facilitate detergent exchange (20 mL, W1). The resin was then washed with buffer A containing 60 mM imidazole (20 mL, W2). The protein was eluted with buffer A containing increasing imidazole concentrations (10 mL of 100 mM, 200 mM and 500 mM each, E1-E3). The fractions were analyzed on SDS-PAGE electrophoresis. The fractions containing PBP2 protein (E1- E3) were combined and concentrated to ~ 10mg/mL using a 50 kD MWCO Amicon Ultra Centrifuge Filter Device (Millipore). The concentrated PBP2 sample was further purified using size-exclusion chromatography with a Superdex S200 column equilibrated in buffer A. The fractions indicating monomeric protein were combined and concentrated using the 50 kD MWCO Amicon Ultra Centrifuge Filter Device, while the concentration was measured on nanodrop using the calculated extinction coefficient of PBP2 [M59-S716]. The final yield was approximately 10 mg per 1.5 L culture. *S. aureus* PBP2 [M59-S716] is referred to PBP2 subsequently. PBP2 S398G, PBP2 D606V, PBP2 P458L were purified using the same protocol. Coomassie stained gel and FPLC spectra of purified PBP2 are shown in Figure 4.2.

4.7.4 LC/MS assay analysis of PBP2 TP activity *in vitro.*

The procedure was modified from our previous report.[36] Native *S. aureus* Lipid II in DMSO (40 μM) was incubated with purified PBP2 (1 μM) in 1x reaction buffer (50 mM HEPES,

pH 7.5, 10 mM $CaCl_2$) in a total of 10 μL reaction volume for 1 h at 25 °C. The reaction was quenched at 95 °C for 5 min, and then treated with mutanolysin (from *Streptomyces globisporus*, Sigma, 1 U) for 1.5 h at 37 °C followed by another 1 U aliquot for 1.5 h. The resulting disaccharides were reduced with sodium borohydride (10 μL of 10 mg/mL solution, 30 min) to resolve the MurNAc anomers. Phosphoric acid (20%, 1.2 μL) was then added to adjust the pH to ~4. The reaction mixture was the lyophilized, redissolved in 12 μL H_2O and subjected to LC/HRMS analysis, conducted with ESI-MS operating in positive mode on a Bruker qTOF mass spectrometer. The instrument was equipped with a Waters Symmetry Shield RP18 column (5 μM, 3.9 x 150 mm) with a matching column guard. The fragments were separated using the following method: 0.5 mL/min H_2O (0.1% formic acid) for 5 min followed by a gradient of 0% acetonitrile (ACN) (0.1% formic acid)/H_2O (0.1% formic acid) to 40% ACN (0.1% formic acid)/H_2O (0.1% formic acid) over 25 min. Molecular ions corresponding to the expected disaccharide fragments were extracted from chromatograms. The reactions with PBP2 S398G or SgtB were carried out using identical conditions (Figure App3.1).

For substrate specificity studies, the reaction was performed similarly except for native Lipid II replaced by either Gly-Lipid II or Gly_3-Lipid II (Figure 4.5).

4.7.5 Degradation of isolated *S. aureus* sacculi

Isolation and degradation of *S. aureus* sacculi were modified from a previous reported procedure.[37] Briefly, an overnight culture of *S. aureus* (2 mL) was centrifuged at 10,000 rpm for 5 min. The pellet was resuspended in 1 mL 0.25% SDS in 0.1 M Tris/HCl, pH~7.0, and boiled at 100 °C for 20 min. The suspension was spun down at 10,000 rpm for 5 min, and the pellet was washed with 1.5 mL H_2O for at least three times to remove SDS. The washed pellet was

resuspended in 1 mL H_2O and let to sonicate in a water bath for 30 min, after which, 500 μL of a solution containing 15 μg/mL DNase, 60 μg/mL RNase in 0.1 M Tris-HCl, pH 6.8 was added. After shaking at 37 °C for 2 h, the mixture was boiled at 100 °C for 3 min to inactivate enzymes, spun down (5 min, 10,000 rpm) and washed with water once (1 mL). To release wall teichoic acid (WTA), the pellet was suspended in 500 μL of 1 M HCl and allowed to shake at 37 °C for 4 h. The pellet was spun down (5 min, 10,000 rpm) and washed with water until pH is 5~6. The pellet was resuspended in a 100 μL digestion buffer of 12.5 mM NaH_2PO_4, pH 5.5, and treated with 10 μL of mutanolysin (5 U/mL in dH_2O), and allowed to shake at 37 °C for 16 h. After digestion, the sample was boiled at 100 °C for 3 min to inactivate enzymes. The sample was spun down (5 min, 10,000 rpm), and added 50 μL $NaBH_4$ (10 mg/mL) at room temperature for 30 min. The sample was adjusted to pH ~4 with 20% phosphoric acid, and lyophilized. The lyophilized materials were resuspended in 500 μL H_2O, and 20 μL was used for LC/MS analysis of the muropeptides. The LC/MS condition is the same as above for PBP2 *in vitro* reaction (Figure 4.3).

4.7.6 Western blot analysis of *S. aureus* PBP2 TP activity

The procedure is similar to the Western blot analysis of Lipid II described in Chapter Two,[22] but PBP4 is replaced by PBP2. Briefly, Lipid II (1 μL of 100 μM stock in DMSO) was incubated with PBP2 (1 μL of 10 μM), BDL (1.5 μL of 20 mM) and 10x reaction buffer (1 μL of 500 mM HEPES, pH 7.5, 100 mM $CaCl_2$) to reach a total volume of 10 μL. The reaction was incubated at room temperature for 15 min, and heat quenched briefly at 100 °C for 1 min. Lysostaphin (0.5 μL of 1 mg/mL) was added to the reaction mixture to resolve the cross-linked product. The reaction was incubated at 37 °C with shaking for 3 h. To quench the reaction, 10 μL

of 2x SDS loading buffer was added. 3 μL of the final mixture was loaded onto a 4-20% gradient polyacrylamide gel and let run at 200 V for 40 min. The products were transferred to Immuno-Blot PVDF membrane (BioRad) at 25V fixed 1.3A variable for 15 min, detected by blotting with streptavidin-HRP (1:10000 dilution, Pierce), and visualized using ECL Prime Western Blotting Detection Reagent (GE Ambersham) and Biomax Light Film (Kodak). The same reaction sequence was carried out using PBP2 S398G with or without lysostaphin treatment (Figure 4.4 and Figure App3.2a).

4.7.7 Studies on beta-lactam inhibition of PBP2 TP activity

The above gel-based assay for PBP2 TP activity was modified slightly to enable study of beta-lactam inhibition. Briefly, PBP2 (1 μL of 10 μM) was pre-incubated with varying concentrations of beta-lactams (0 - 50 μM) in a reaction mixture containing BDL (1.5 μL of 20 mM) and 10x reaction buffer (1 μL of 500 mM HEPES, pH 7.5, 100 mM $CaCl_2$) for 5 min (unless otherwise noted). Lipid extract (1 μL of 100 μM stock in DMSO) was then added to the reaction and let to incubate at room temperature for 15 min (unless otherwise noted), and heat quenched briefly at 100 °C for 1 min. Lysostaphin (0.5 μL of 1 mg/mL) was added to the reaction mixture to resolve the cross-linked product. The reaction was incubated at 37 °C with shaking for 3 h. To quench the reaction, 10 μL of 2x SDS loading buffer was added. Western blot analysis was performed as described above (Figure 4.4 and Figure App3.2).

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Appendix One: Mass spectrometry (MS) analysis for studies in Chapter Two

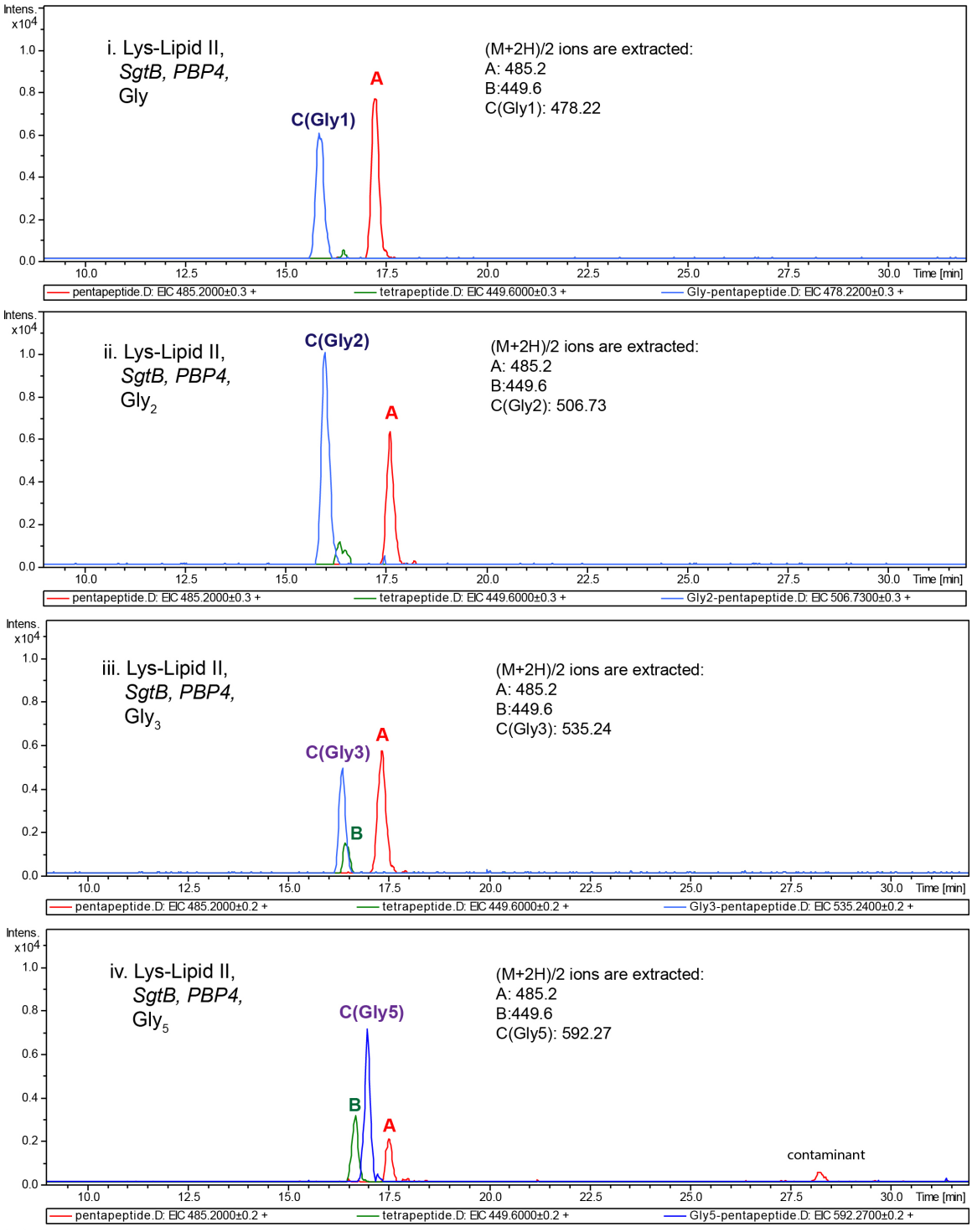


Figure App1.1. *S. arureus* PBP4 shows TP activity *in vitro*. LC/MS chromatograms showing oligoglycine incorporation by *S. aureus* PBP4.

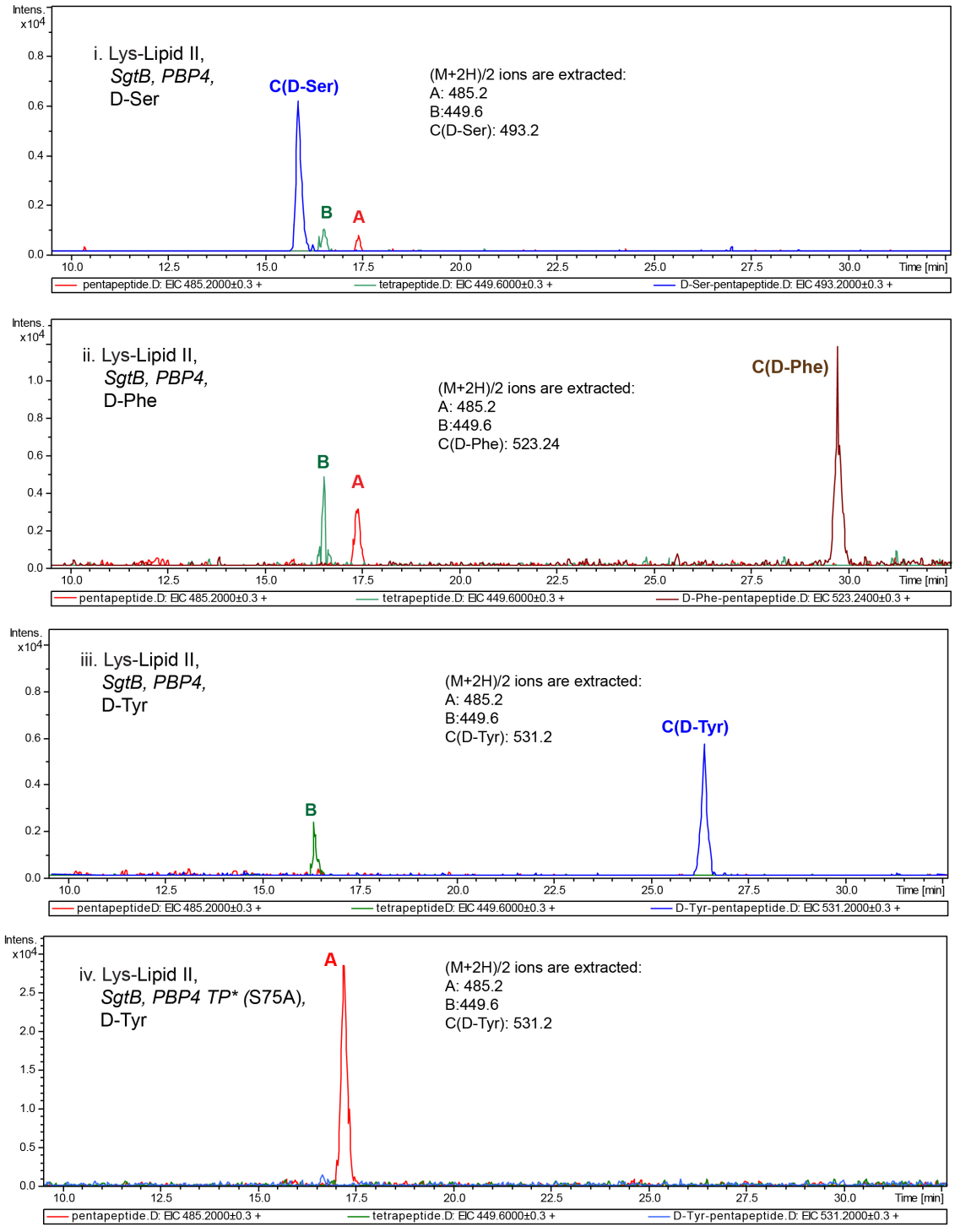


Figure App1.2. *S. arureus* PBP4 shows TP activity *in vitro*. LC/MS chromatograms showing D-amino acid incorporation by *S. ausreus* PBP4.

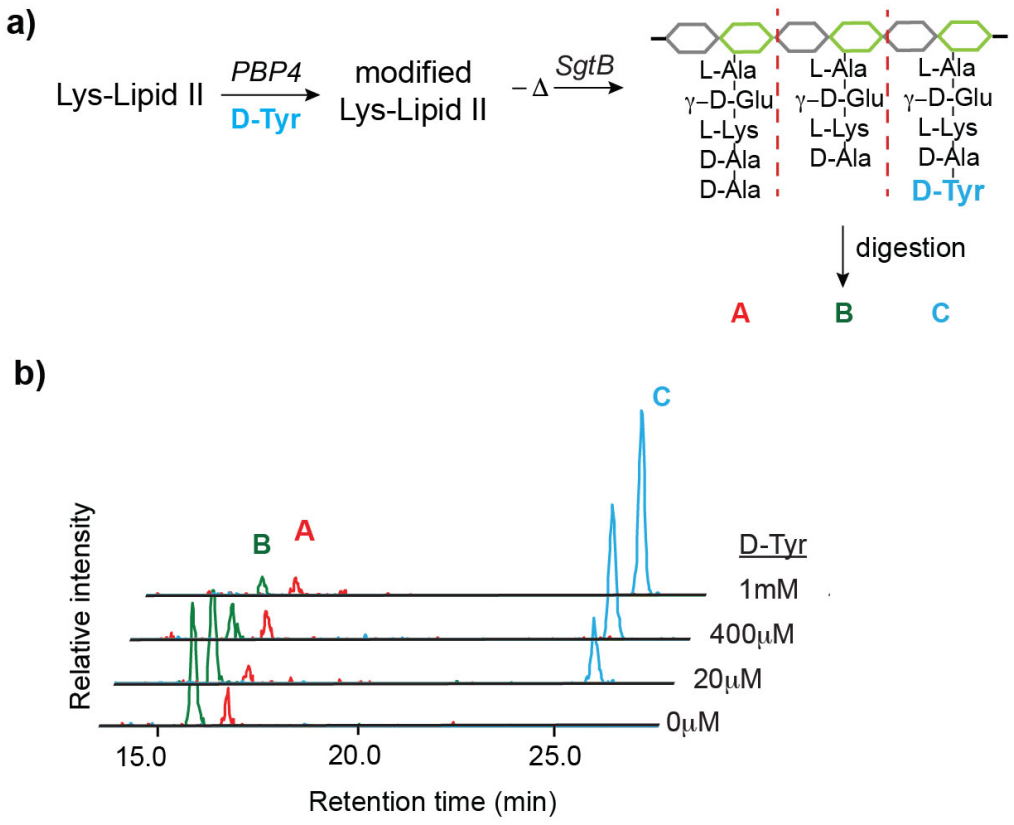


Figure App1.3. Analysis of *S. aureus* PBP4 activities at varying concentrations of D-Tyr nucleophile.

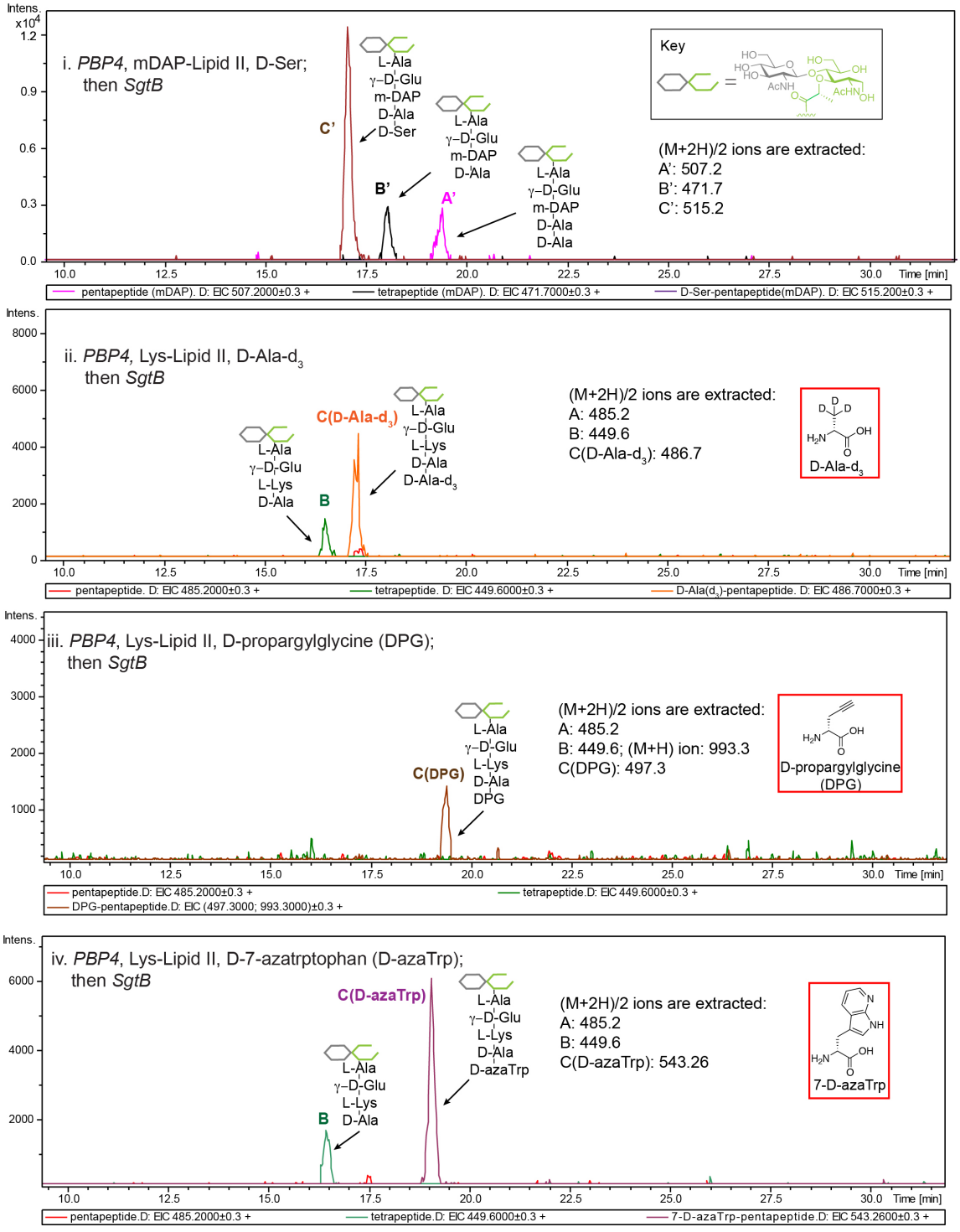


Figure App1.4. *S. aureus* PBP4 efficiently incorporates D-amino acids into *m*-Dap-Lipid II and Lys-Lipid II *in vitro*.

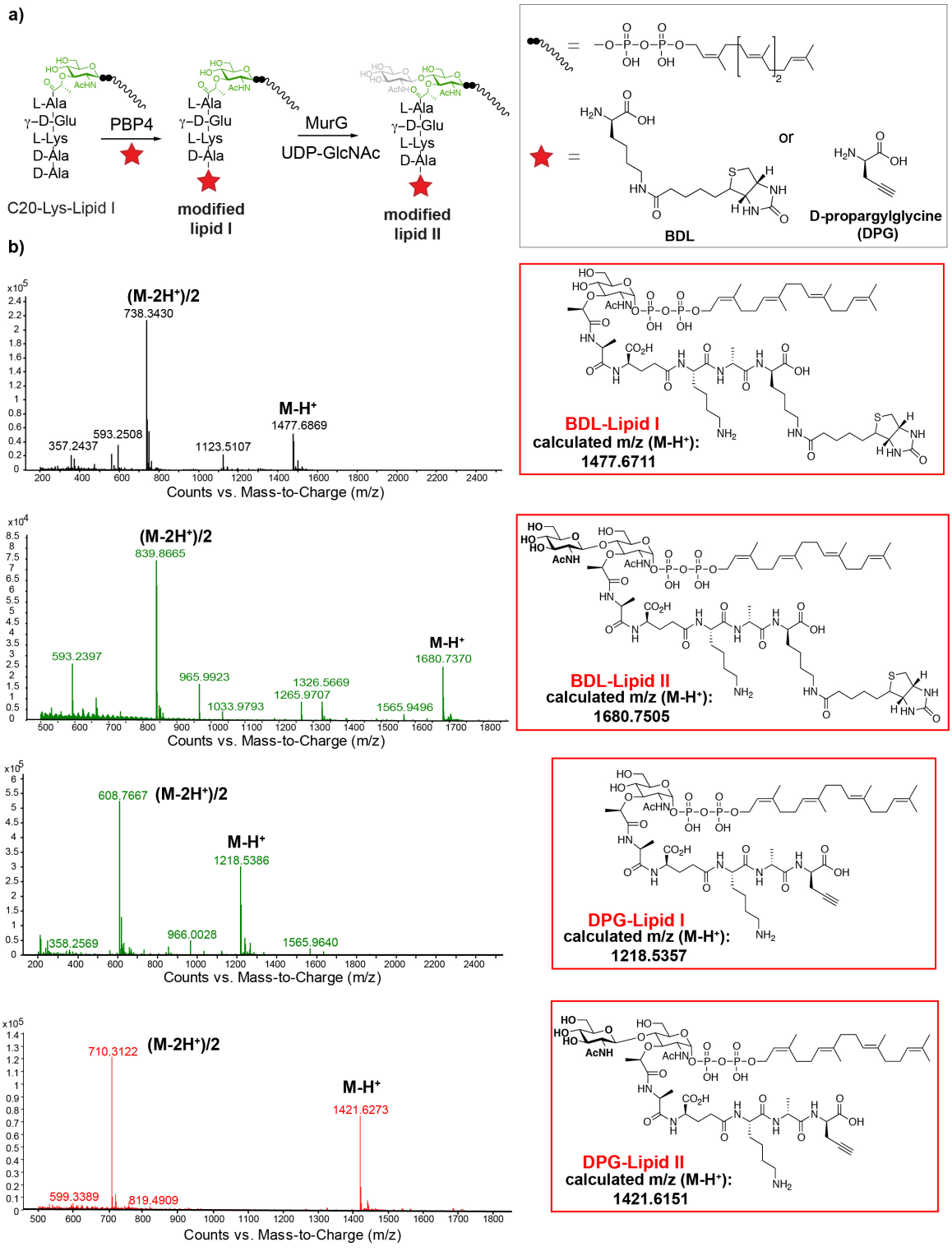


Figure App.1.5. *S. aureus* PBP4 incorporates functionalized D-amino acids into Lys-Lipid I and Lys-Lipid II *in vitro*.

Appendix Two: NMR and MS data for compounds in Chapter Three

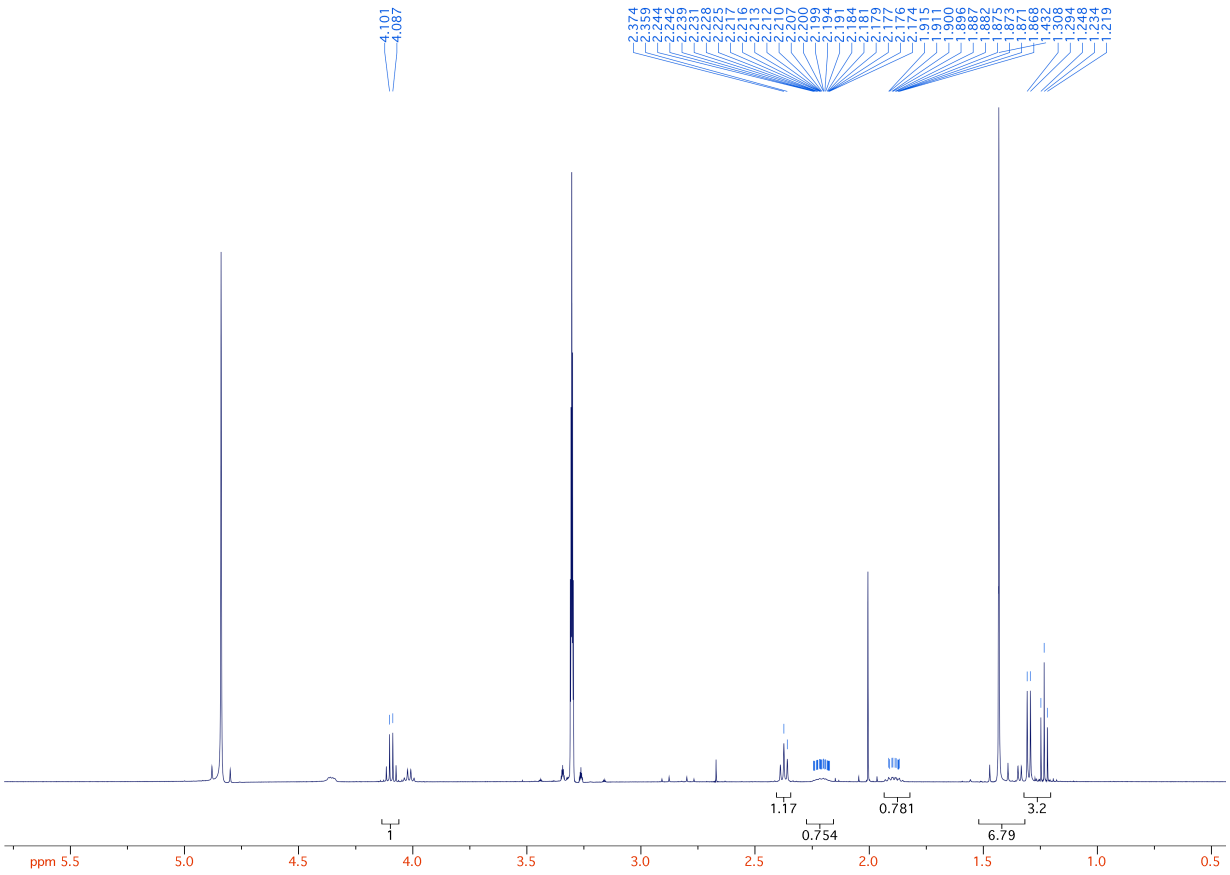


Figure App2.1. ^{1}H NMR of Boc-L-Ala-D-isoGln-OH.

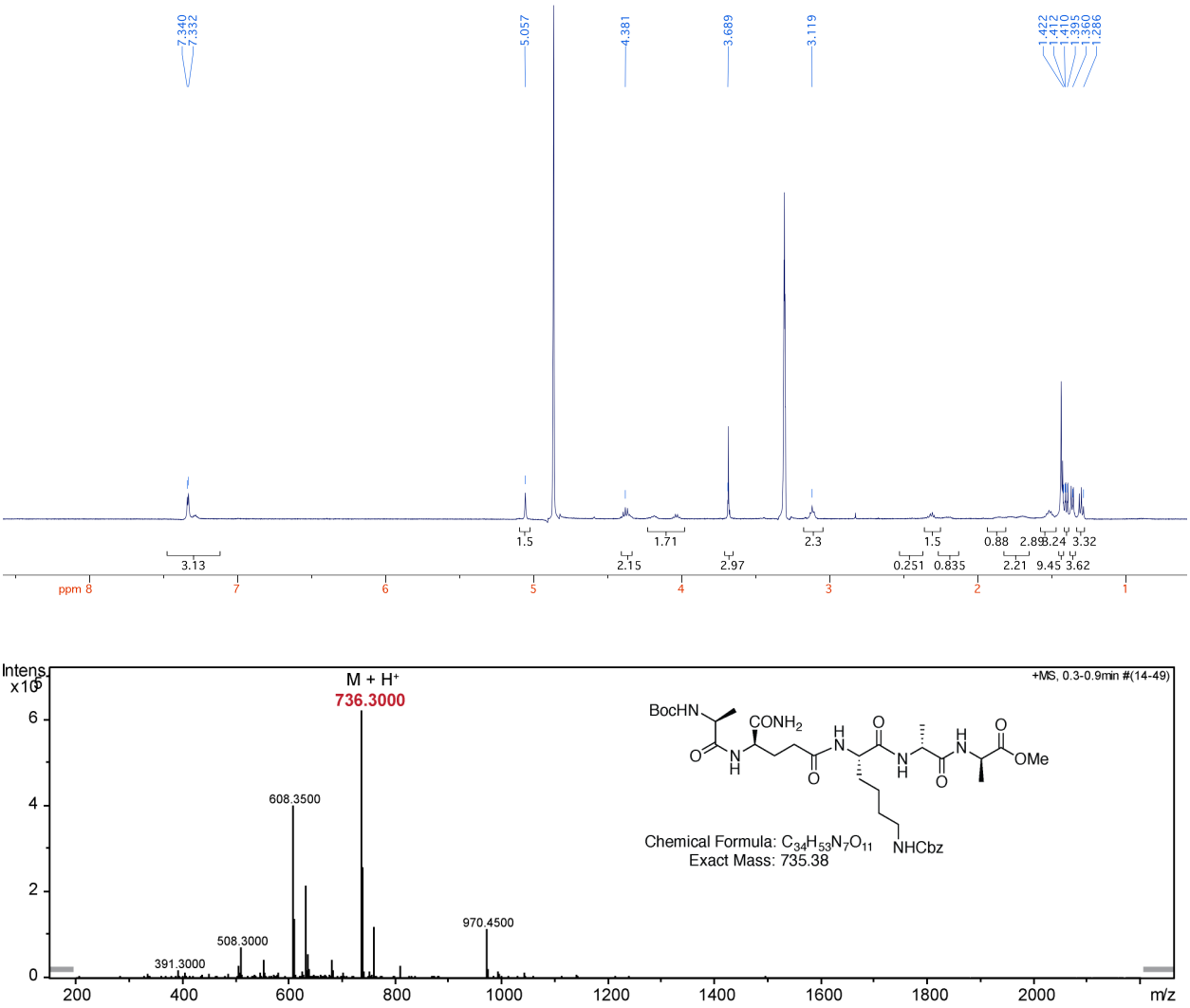
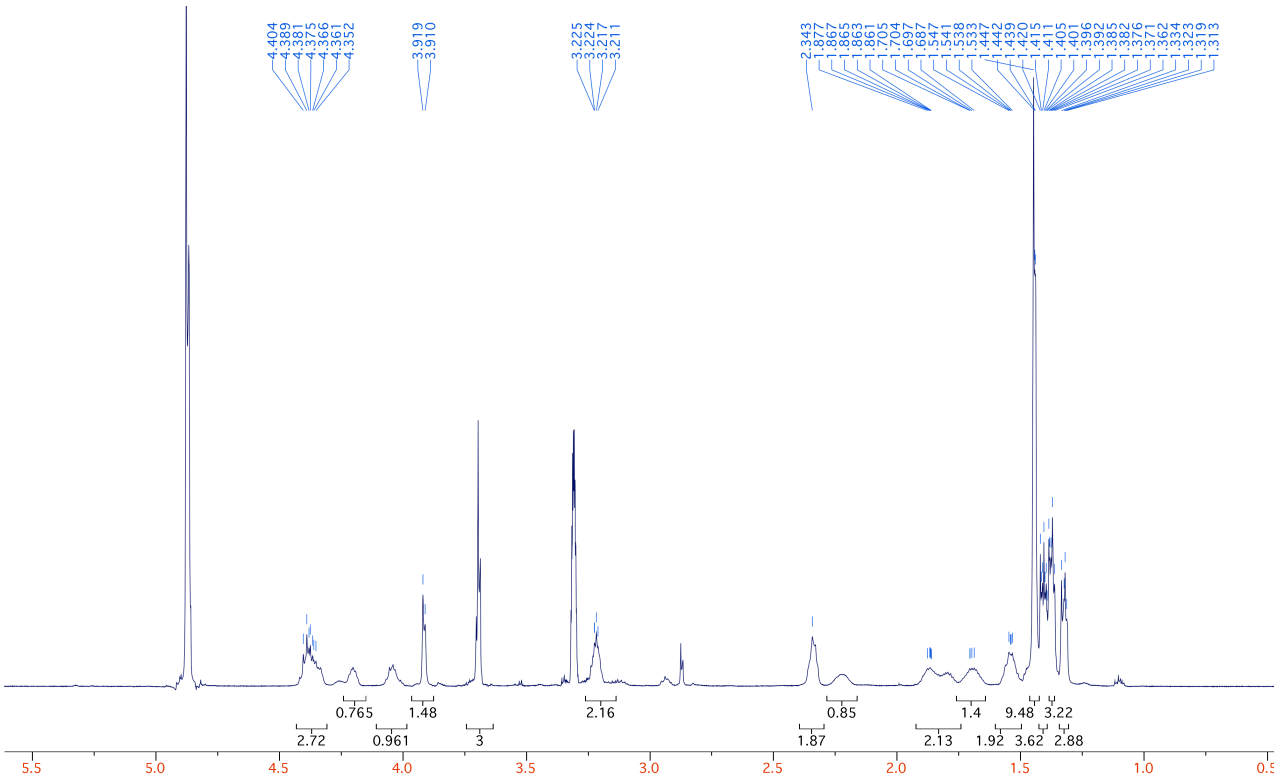


Figure App2.2. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Ala-D-*iso*Gln-L-Lys(Cbz)-D-Ala-D-Ala-OMe (**1**).



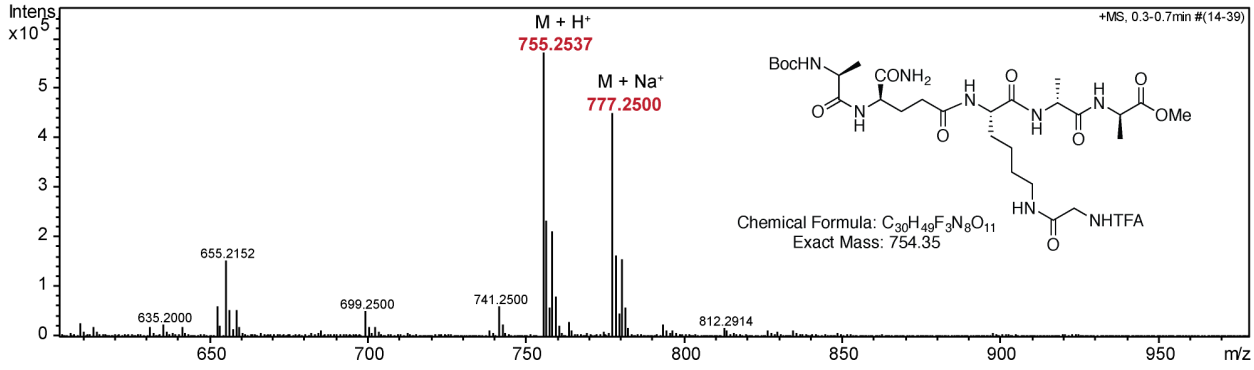


Figure App2.3. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-TFA)-D-Ala-D-Ala-OMe (**3**).

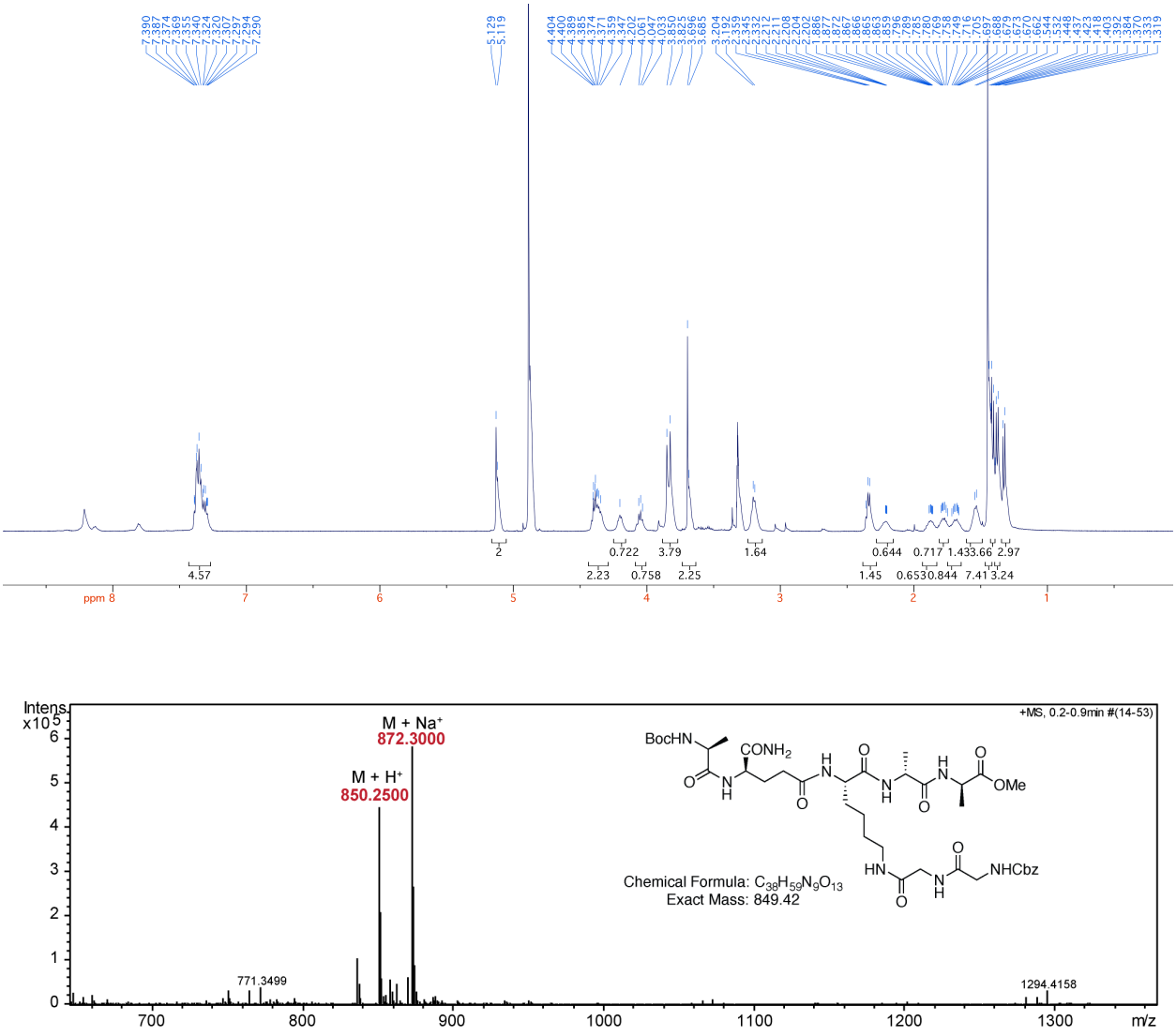
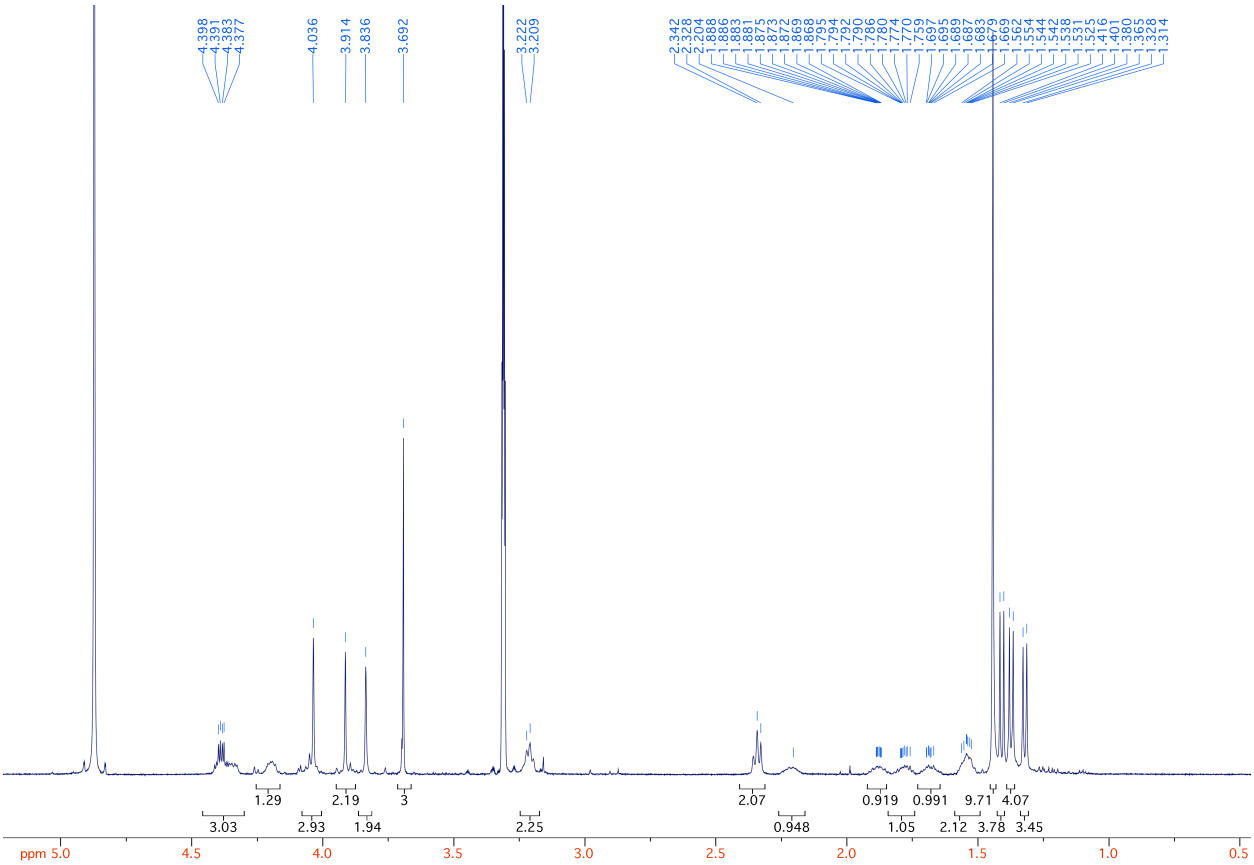


Figure App2.4. [1]H NMR (top) and LRMS (bottom) of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (**4**).



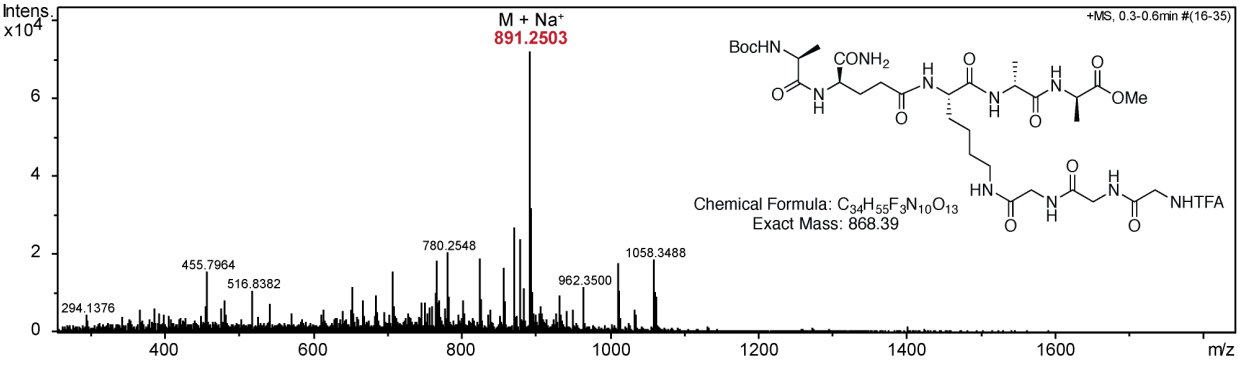


Figure App2.5. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**5**).

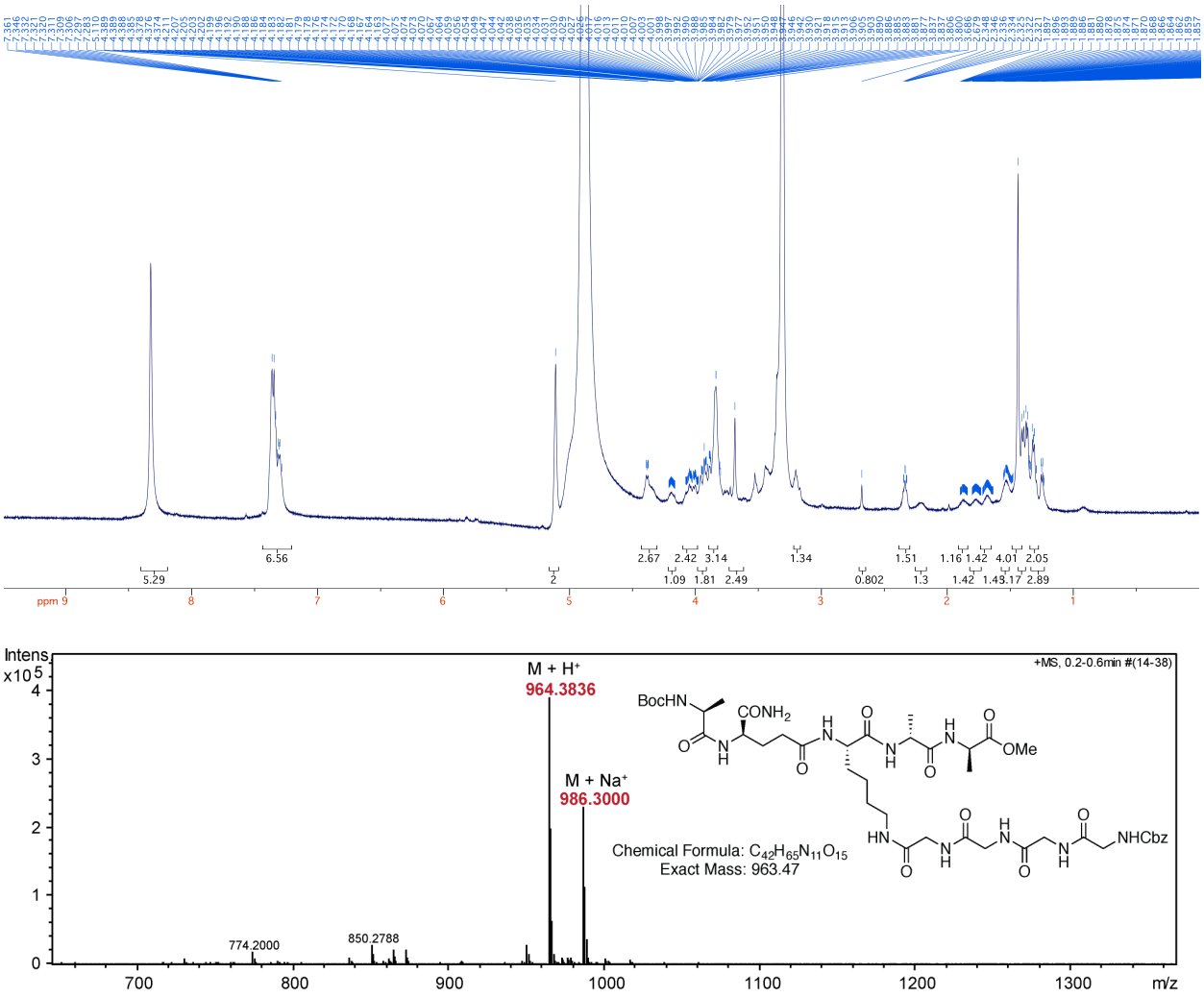


Figure App2.6. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Cbz)-D-Ala-D-Ala-OMe (**6**).

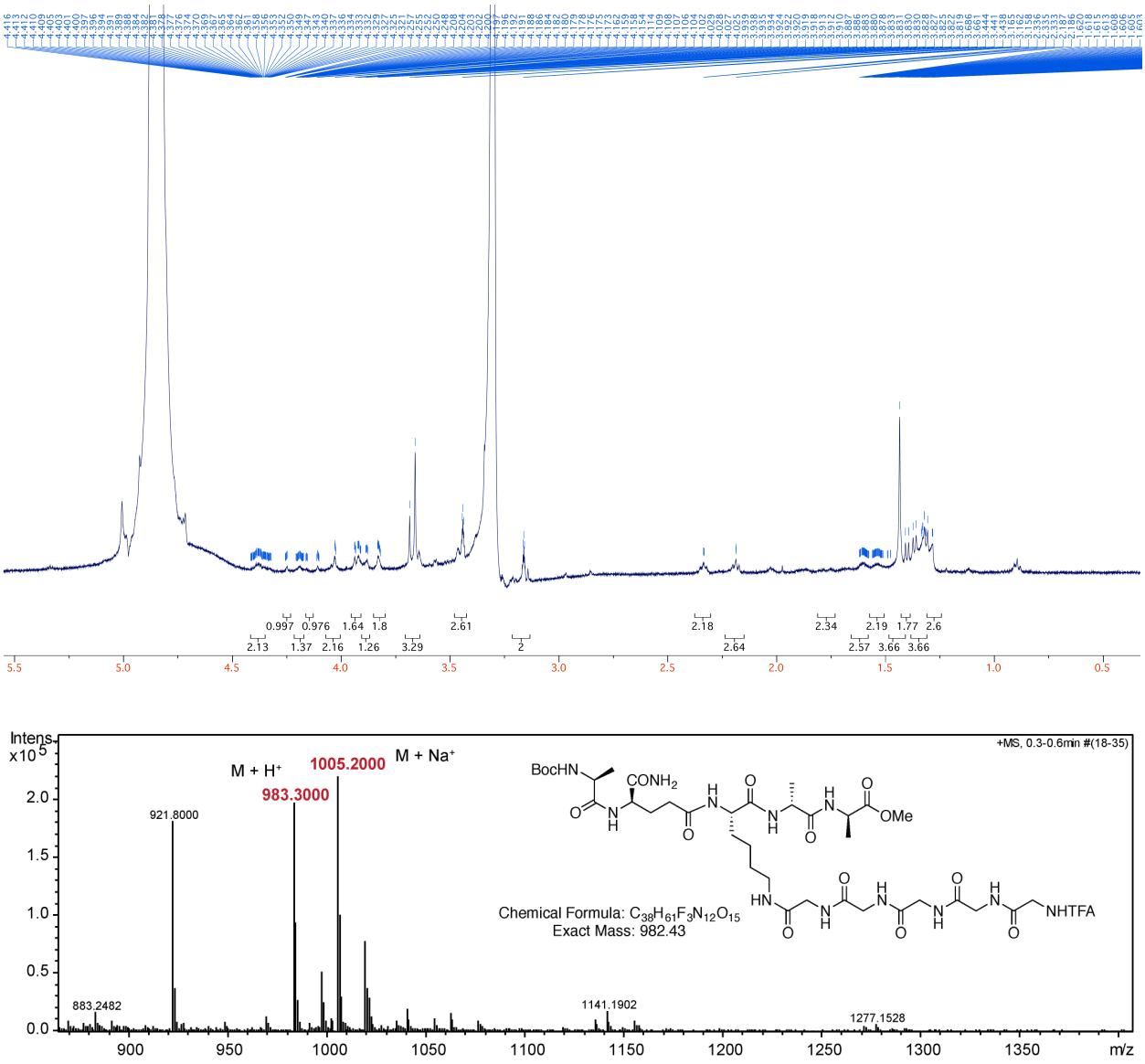


Figure App2.7. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Ala-D-*iso*Gln-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**7**).

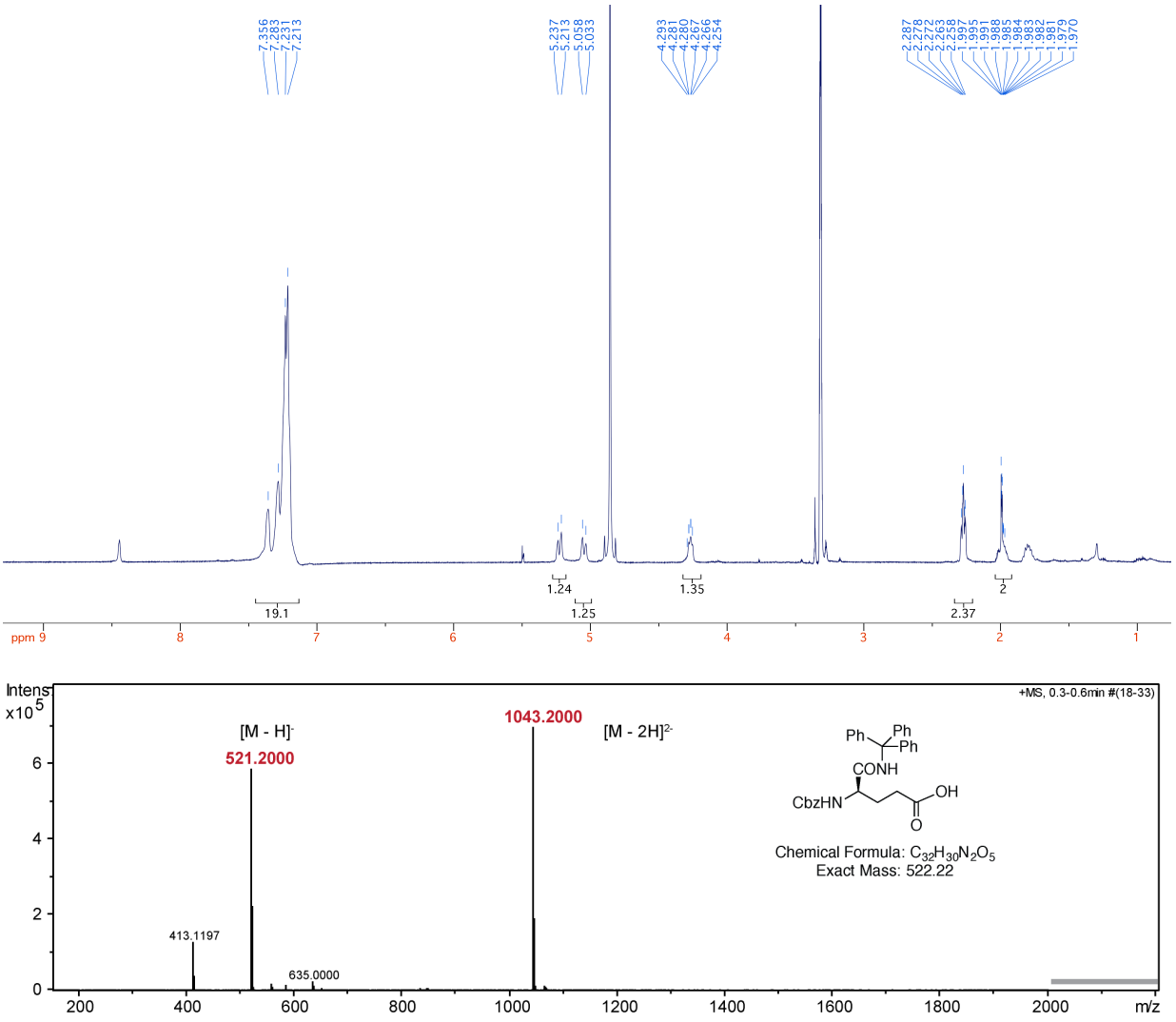


Figure App2.8. ^{1}H NMR (top) and LRMS (bottom) of Cbz-D-isoGln(Trt)-OH.

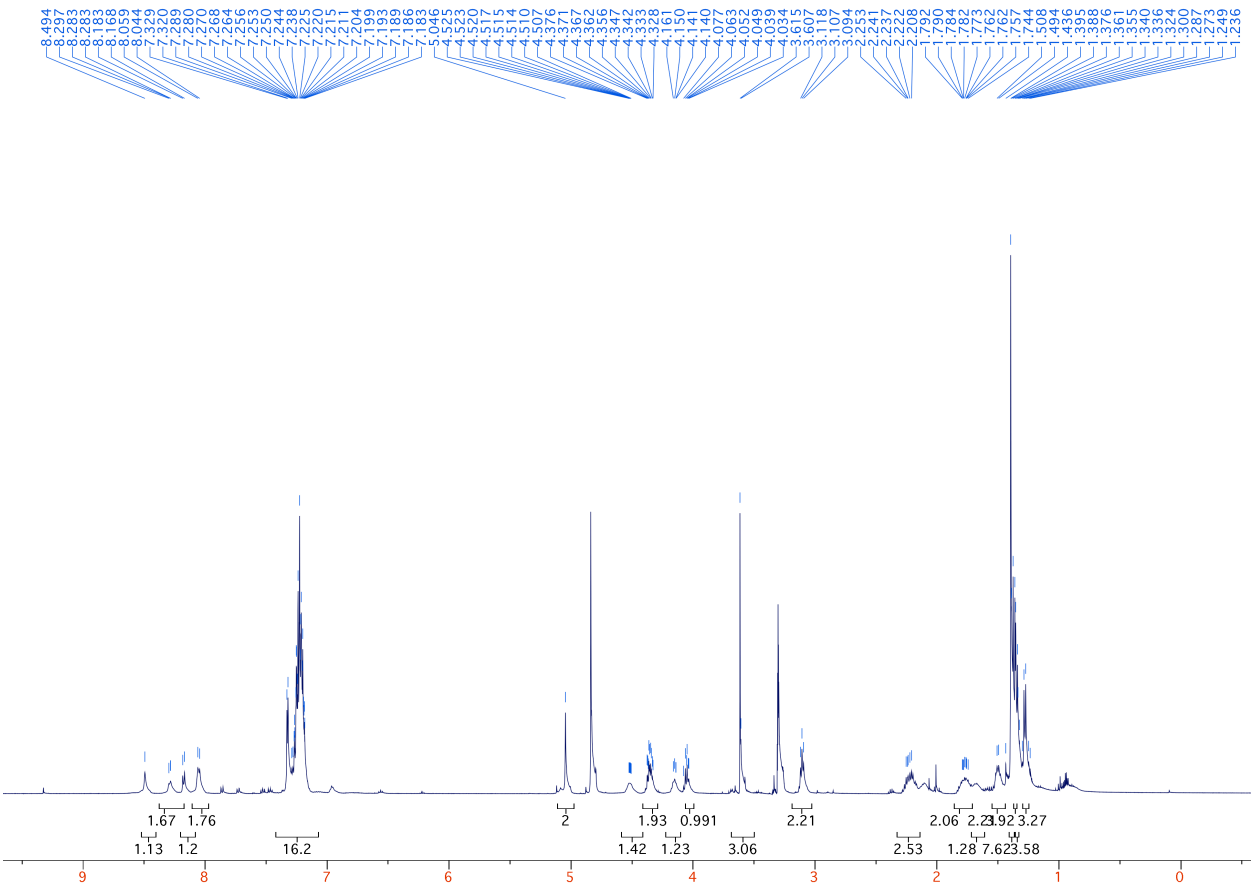


Figure App2.9. ^{1}H NMR of Boc-L-Ala-D-*iso*Gln(Trt)-L-Lys(Cbz)-D-Ala-D-Ala-OMe (**9**).

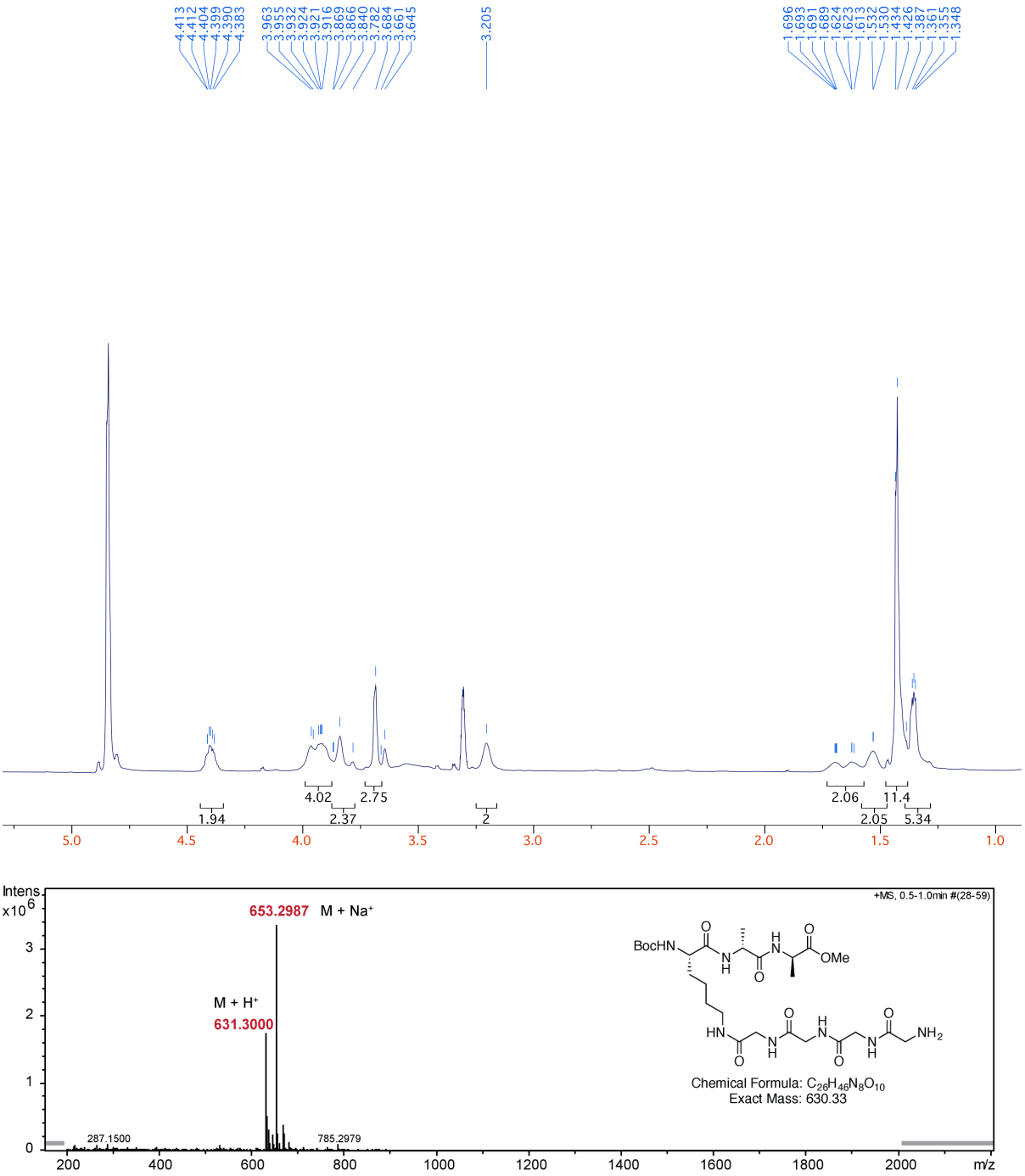


Figure App2.10. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Lys(-Gly-Gly-Gly-Gly-NH_2)-D-Ala-D-Ala-OMe (**10**).

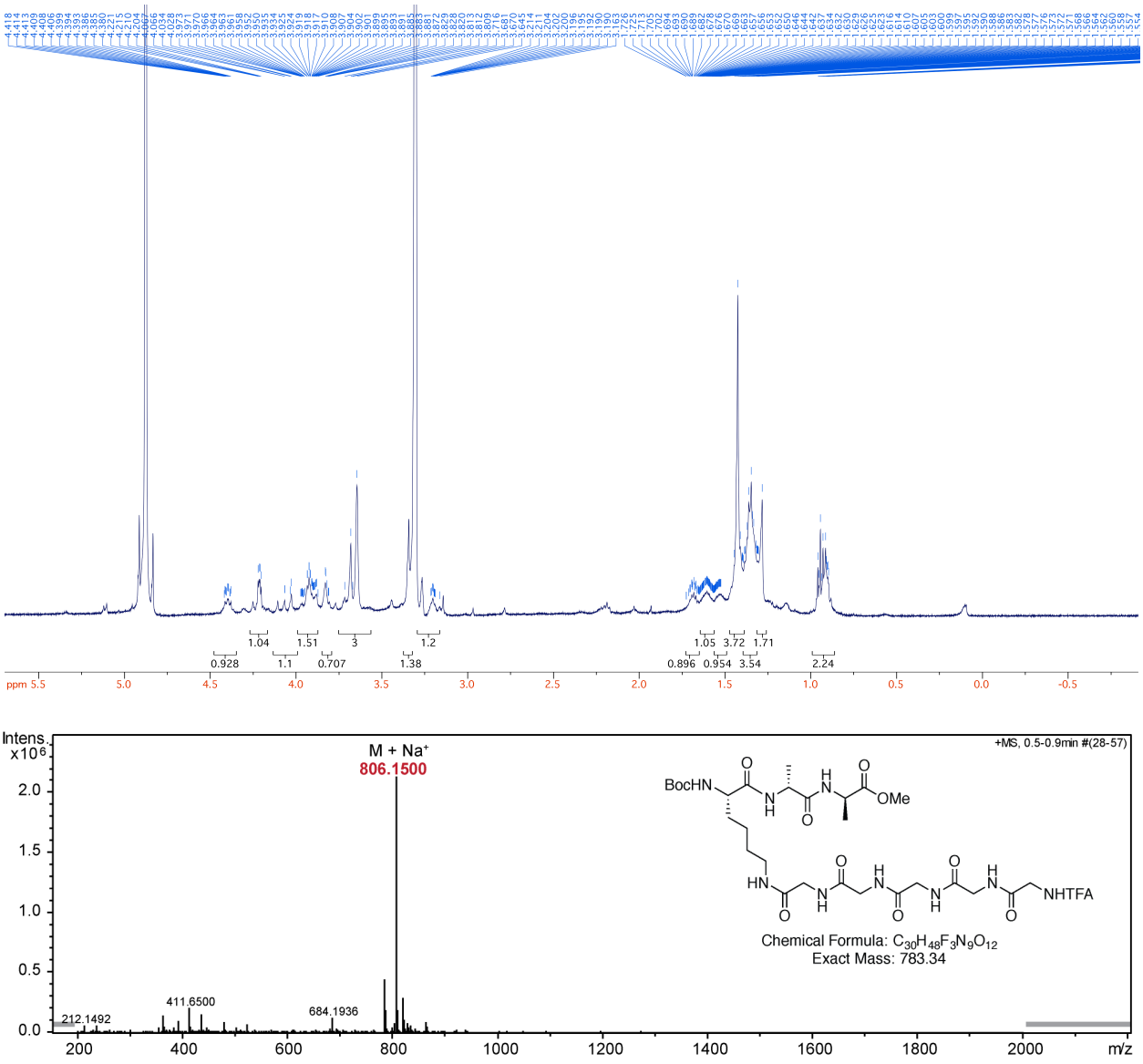


Figure App2.11. ^{1}H NMR (top) and LRMS (bottom) of Boc-L-Lys(-Gly-Gly-Gly-Gly-Gly-TFA)-D-Ala-D-Ala-OMe (**11**).

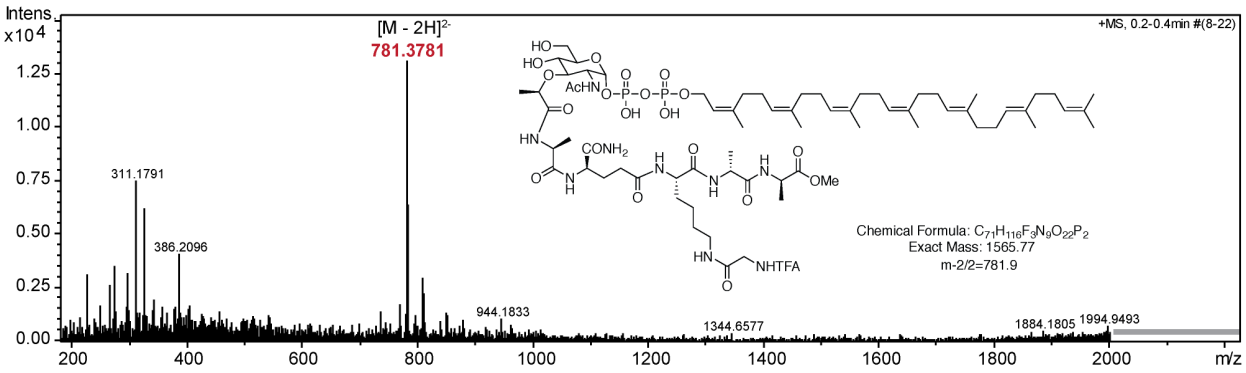


Figure App2.12. LRMS of protected Gly-Lipid I.

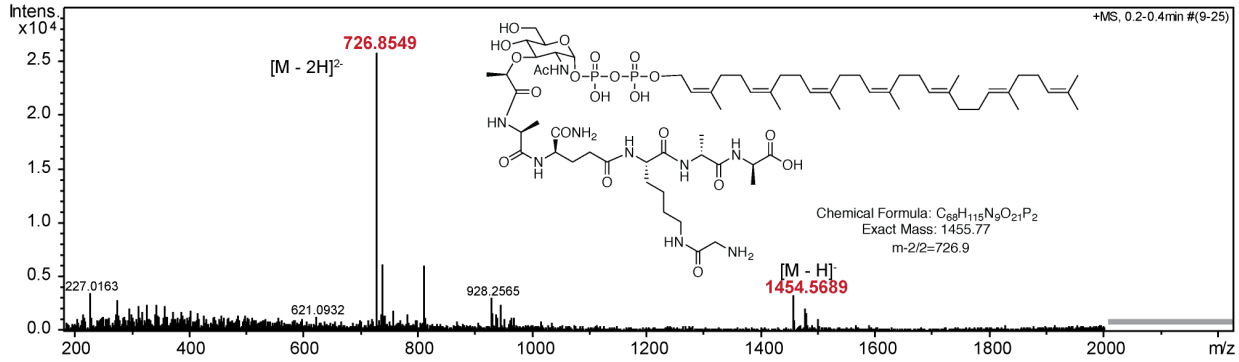
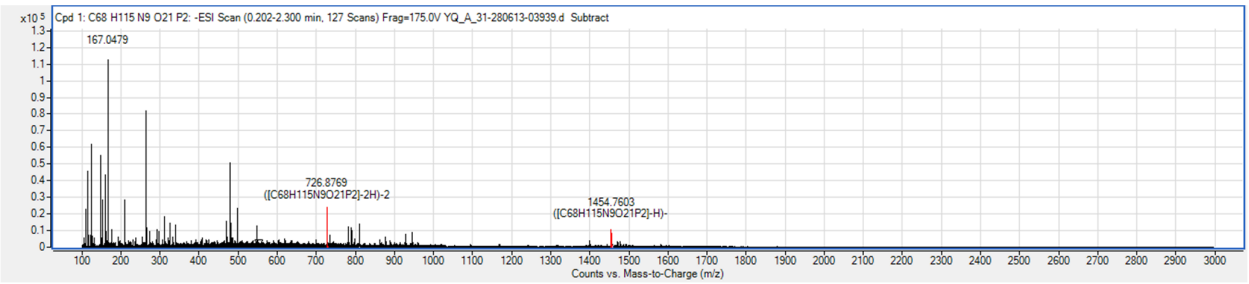
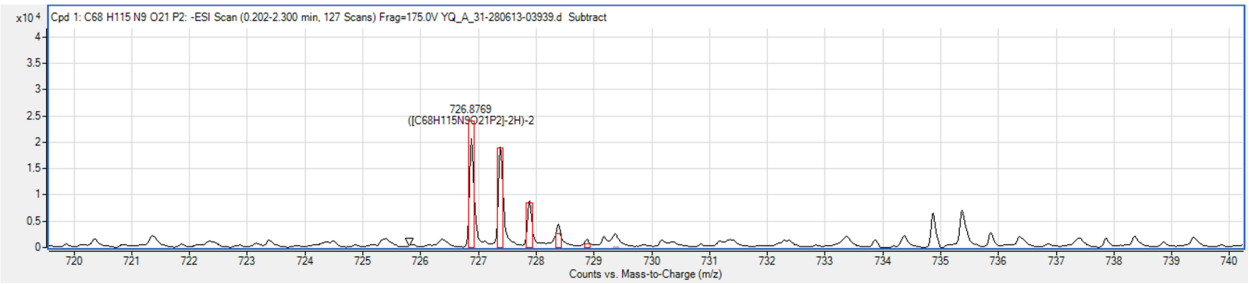
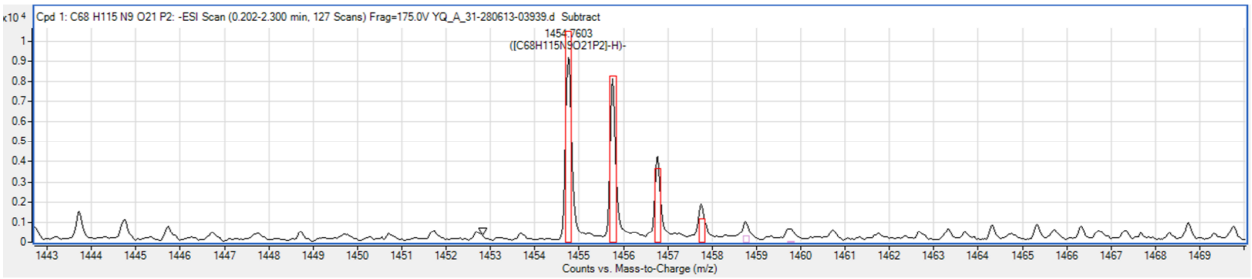


Figure App2.13. LRMS of Gly-Lipid I (**12**).







Ion	Theoretical	Observed	Relative Error ppm
[M-H]-	1454.7610	1454.7603	0.48
[M-2H]2-	726.8769	726.8769	0

Figure App2.14. HRMS of Gly-Lipid I (**12**).

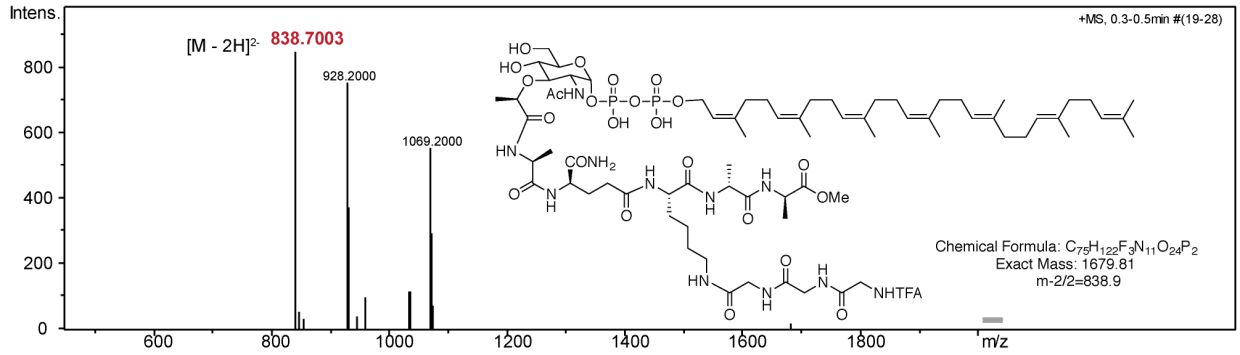


Figure App2.15. LRMS of protected Gly_3-Lipid I.

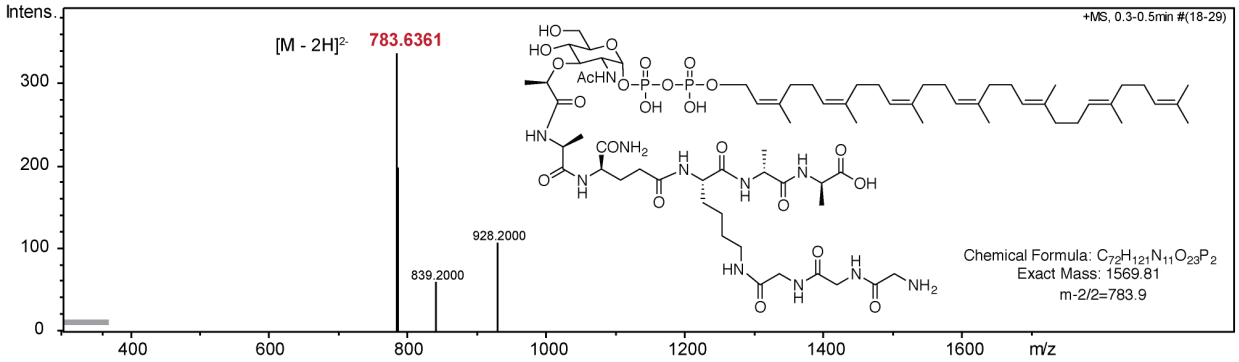
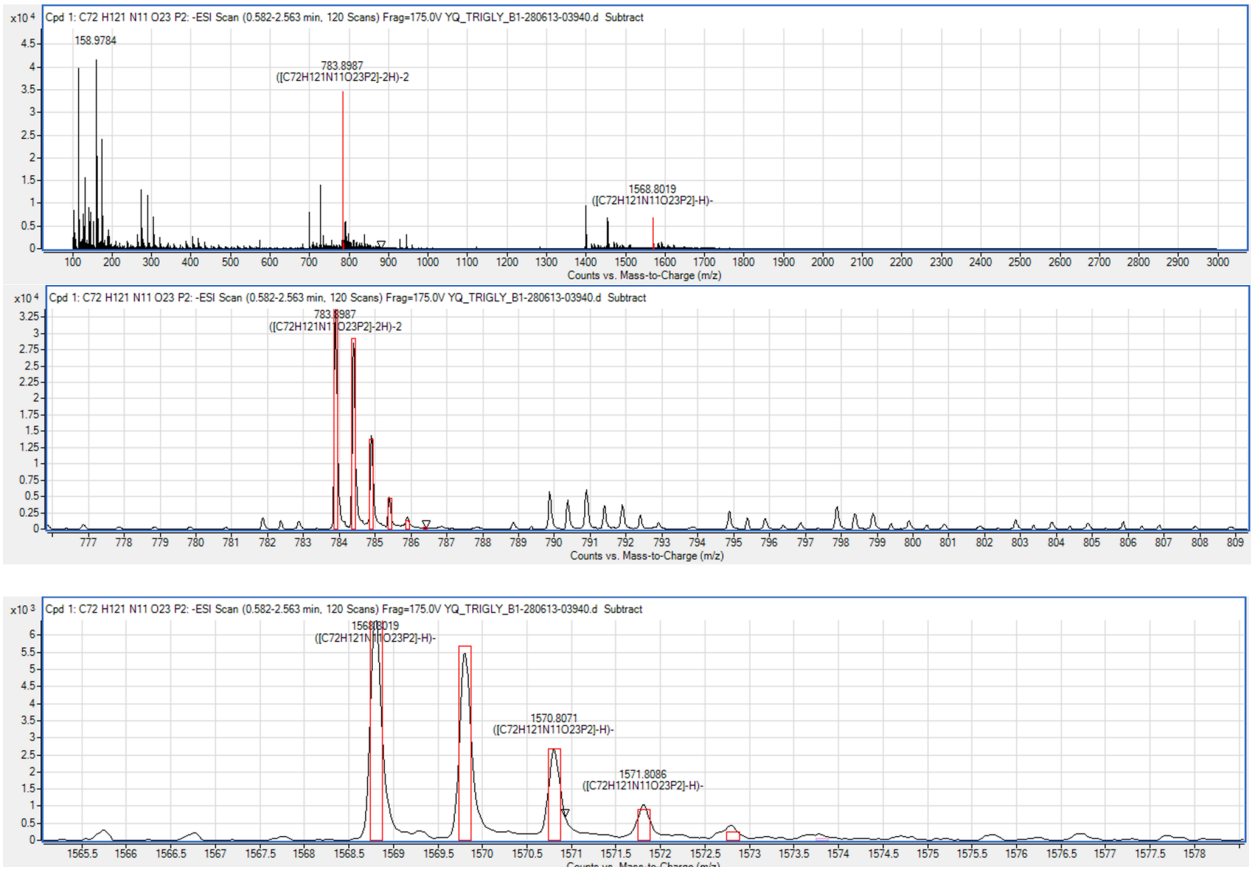


Figure App2.16. LRMS of Gly_3-Lipid I (**13**).



Ion	Theoretical	Observed	Relative Error ppm
[M-H]-	1568.8039	1568.8019	1.2 ppm
[M-2H]2-	783.8983	783.8987	0.5 ppm

Figure App2.17. HRMS of Gly_3-Lipid I (**13**).

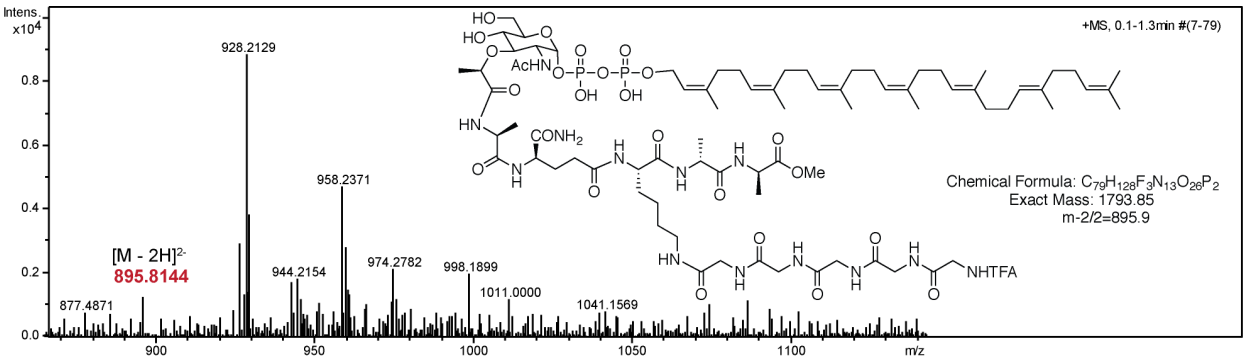
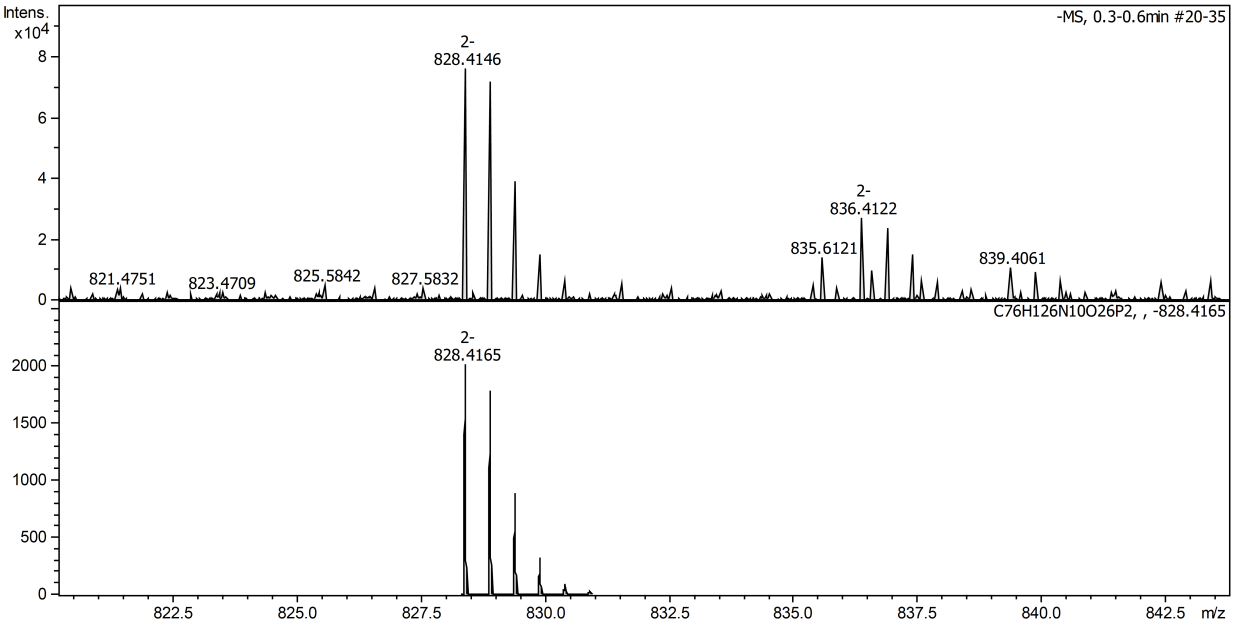


Figure App2.18. LRMS of protected Gly_5-Lipid I (**14**).



Ion	Theoretical	Observed	Relative Error
[M-2H]2-	828.4165	828.4146	2.29 ppm

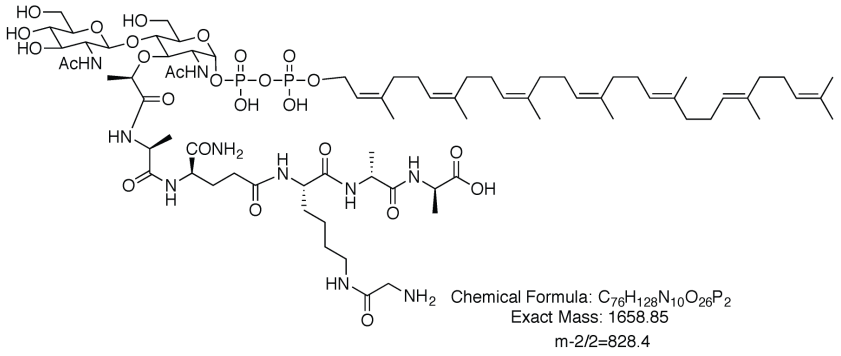
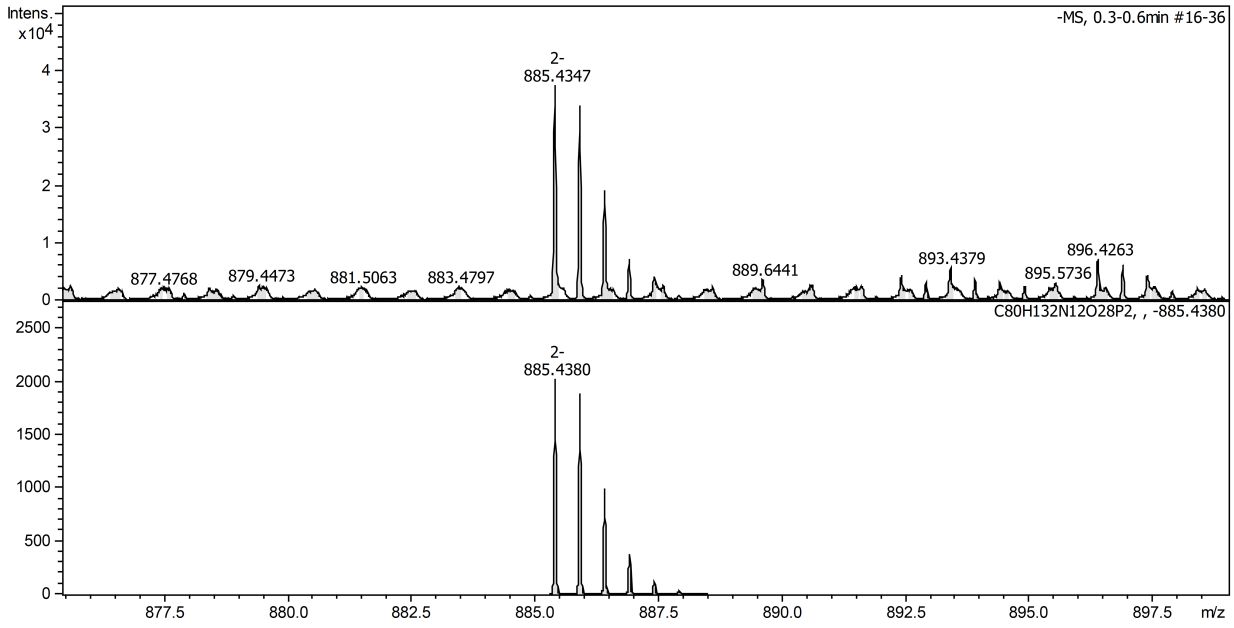


Figure App2.19. HRMS of Gly-Lipid II (**15**).



Ion	Theoretical	Observed	Relative Error
[M-2H]2-	885.4380	885.4347	3.72 ppm

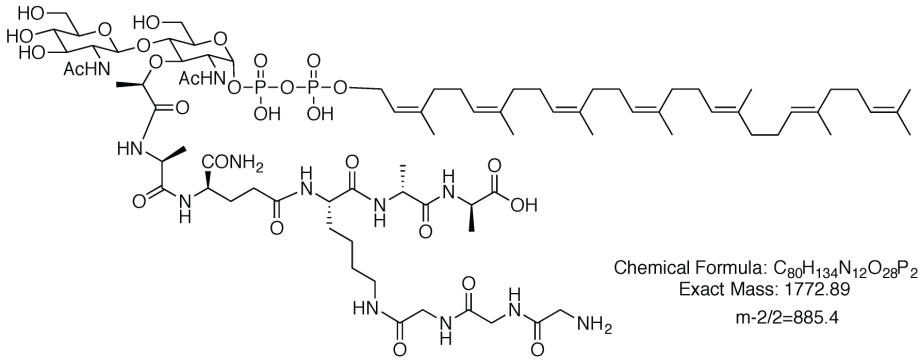


Figure App2.20. HRMS of Gly_3-Lipid II (**16**).

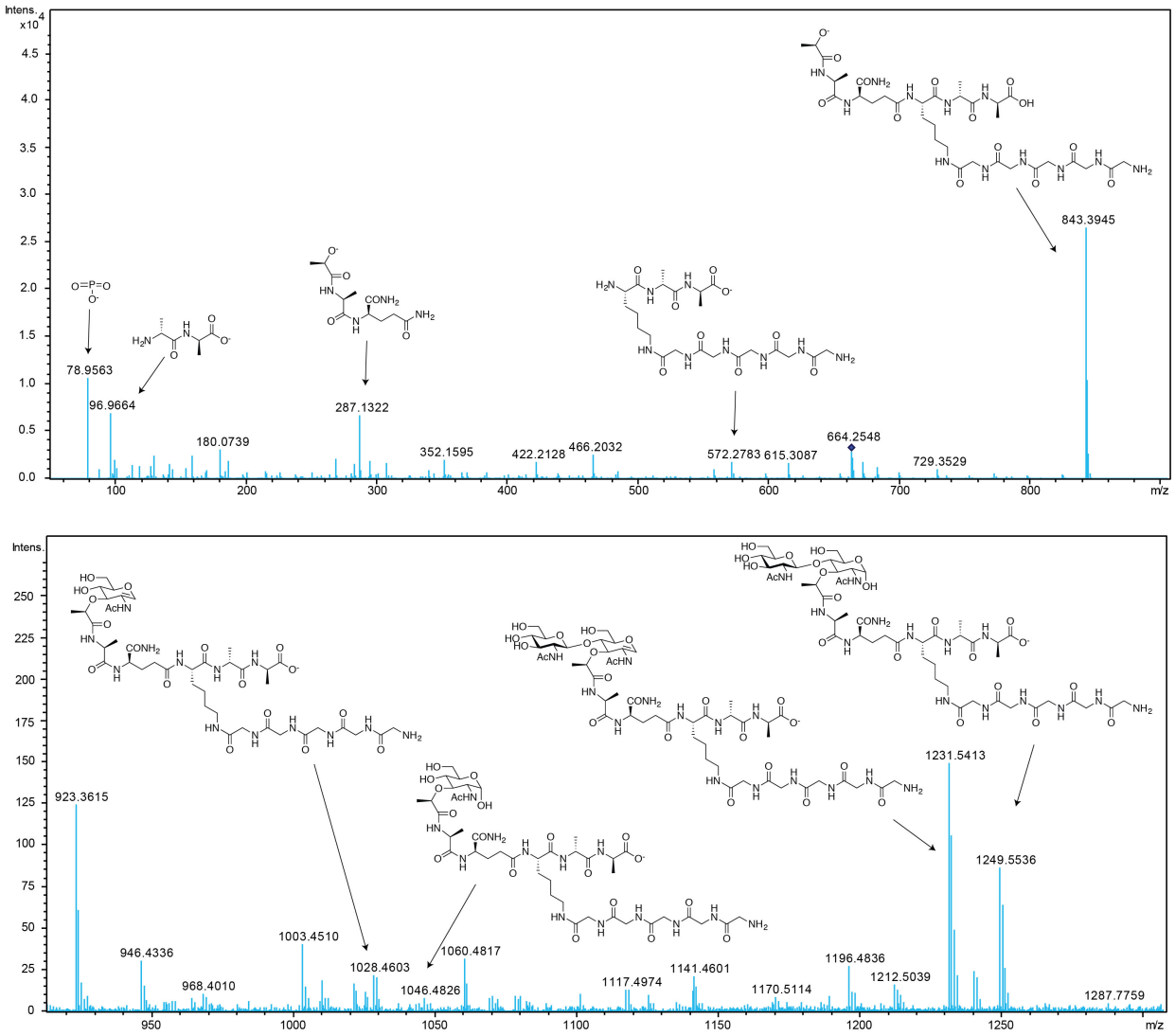


Figure App2.21. LC/MS/MS of delipidated *S. aureus* Lipid II.

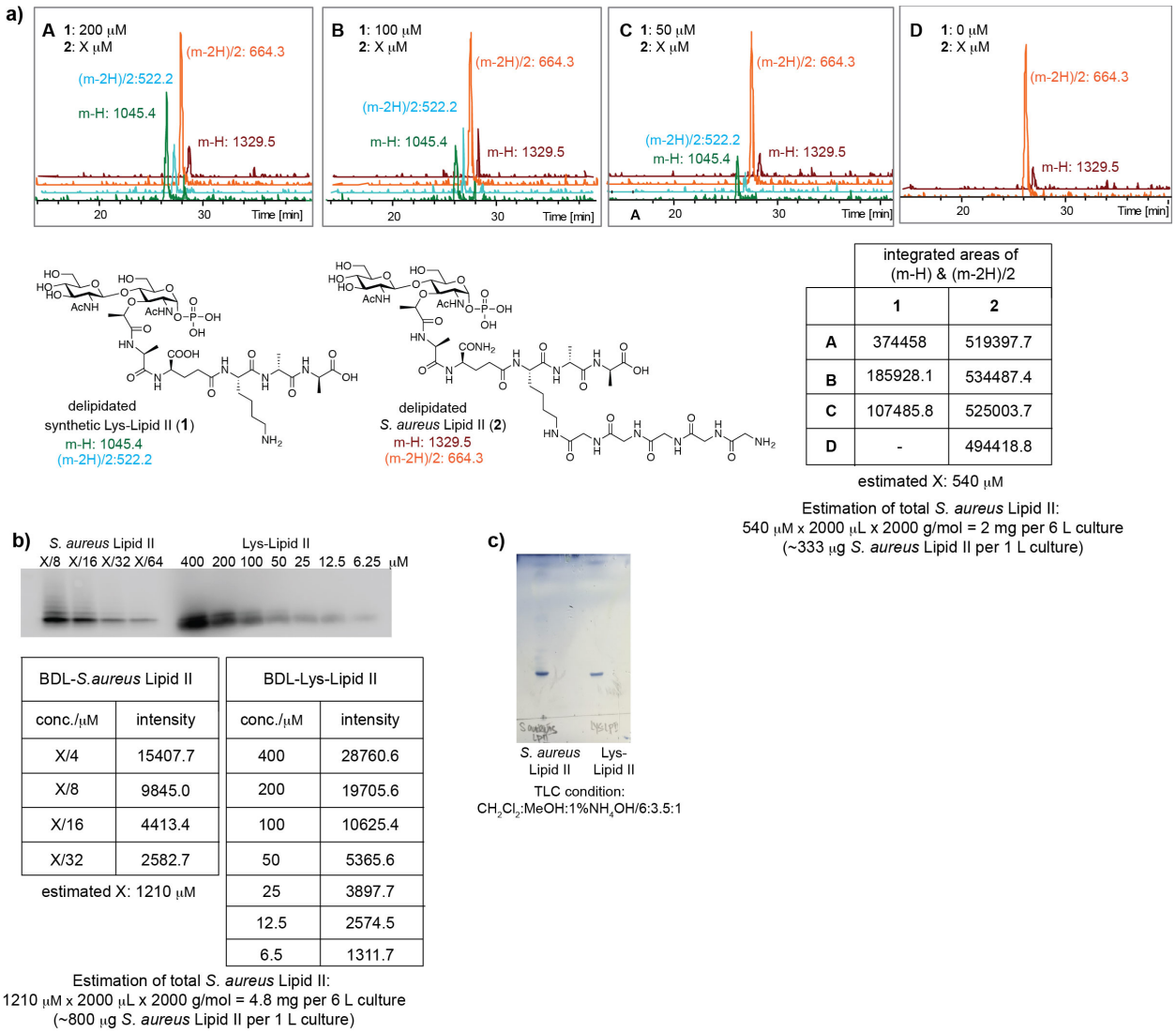


Figure App2.22. Estimation of the amount of *S. aureus* Lipid II isolated from cells. Synthetic Lys-Lipid was used as a standard. **a)** Known amount of Lys-Lipid II was mixed with the *S. aureus* Lipid II sample for lipid cleavage and LC/MS analysis. A Lys-Lipid II standard curve was used to calculate *S. aureus* Lipid II concentration. The following equation was used: amount of *S. aureus* Lipid II = concentration x volume x Mw of *S. aureus* Lipid II. In this manner, 300 μg *S. aureus* Lipid II (from 1 L culture) was estimated. **b)** Serial dilutions of *S. aureus* Lipid II and Lys-Lipid II samples were prepared, biotinylated, and subjected to Western blot analysis. The band intensity was quantified on ImageJ. The standard curve of Lys-Lipid II was used to calculate the concentration of *S. aureus* Lipid II sample. 800 μg Lipid II (from 1 L culture) was estimated. **c)** *S. aureus* Lipid II appeared to contain predominant one species on TLC. Cerium Ammonium Molybdate (CAM) stain was used.

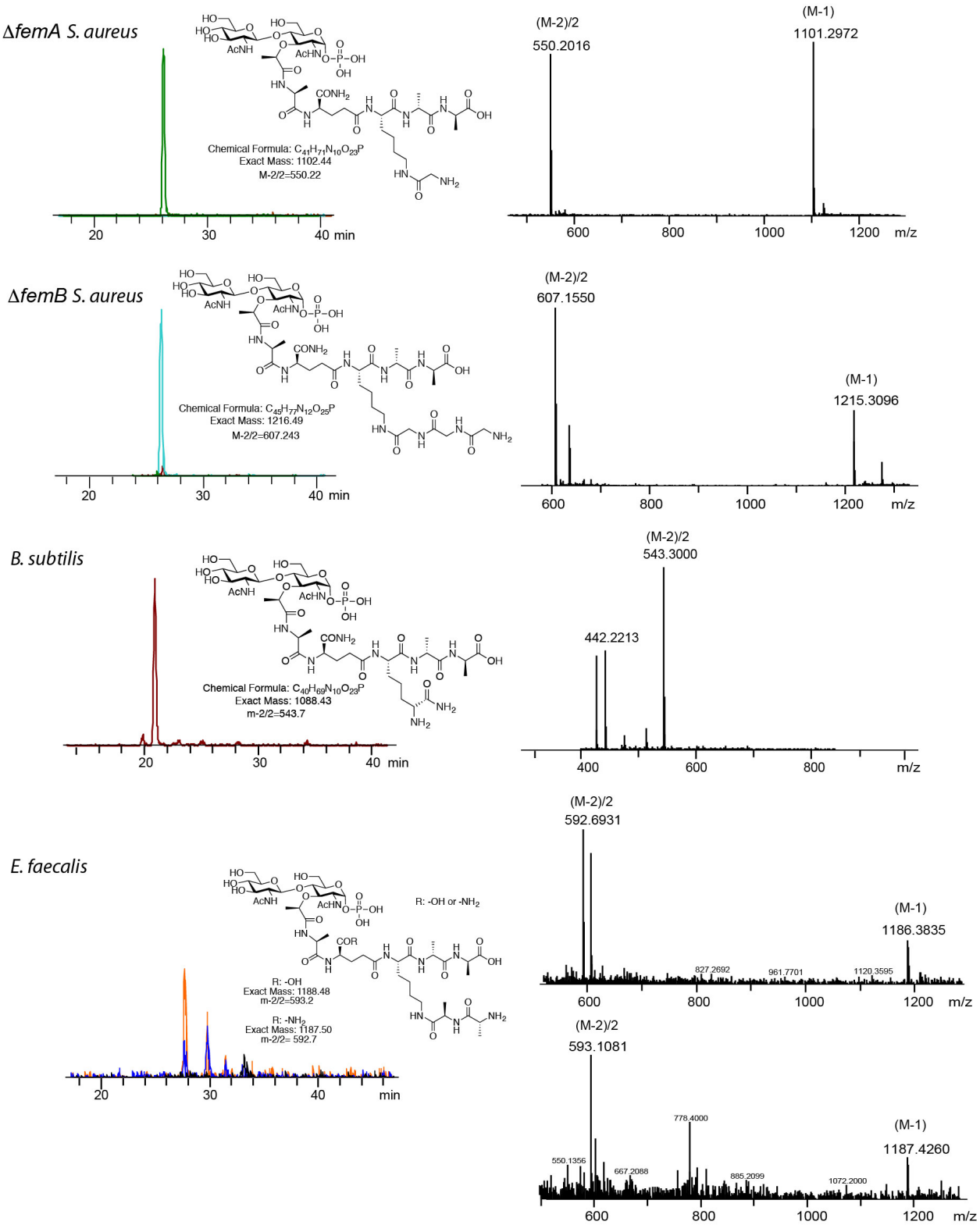


Figure App2.23. LC/MS analysis of delipidated Lipid II from different bacteria: Δ*femA S. aureus*; Δ*femB S. aureus*; *B. subtilis*; *E. faecalis*

Appendix Three: MS and Western blots data for studies in Chapter Four

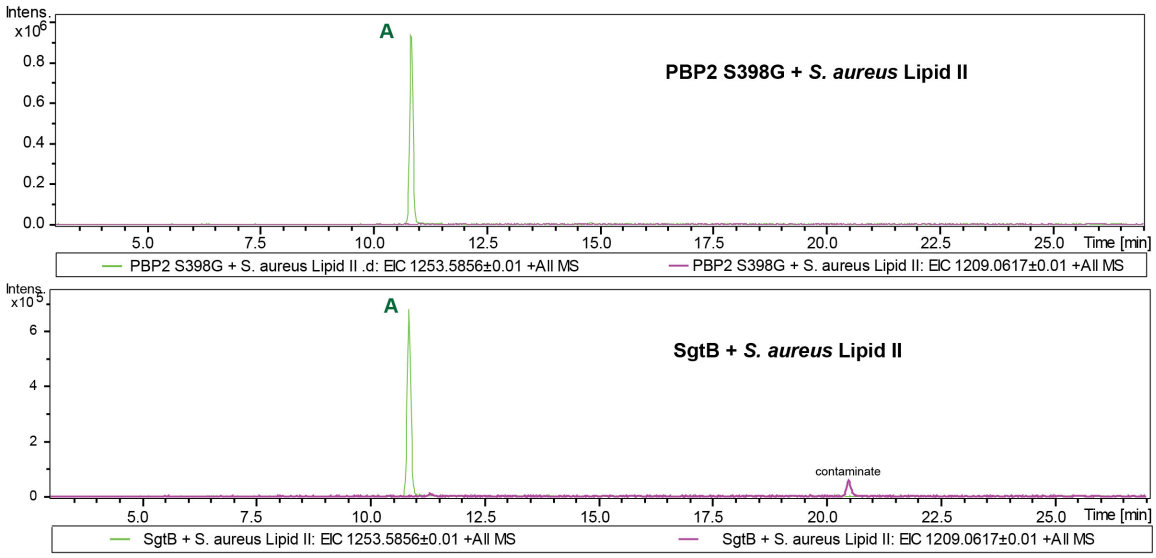


Figure App3.1. The TP domain of *S. aureus* PBP2 is responsible for PG cross-linking. *S. aureus* PBP2 TP inactive mutant (S398G) and SgtB, a monofunctional glycosyltransferase, do not produce dimer muropeptide products.

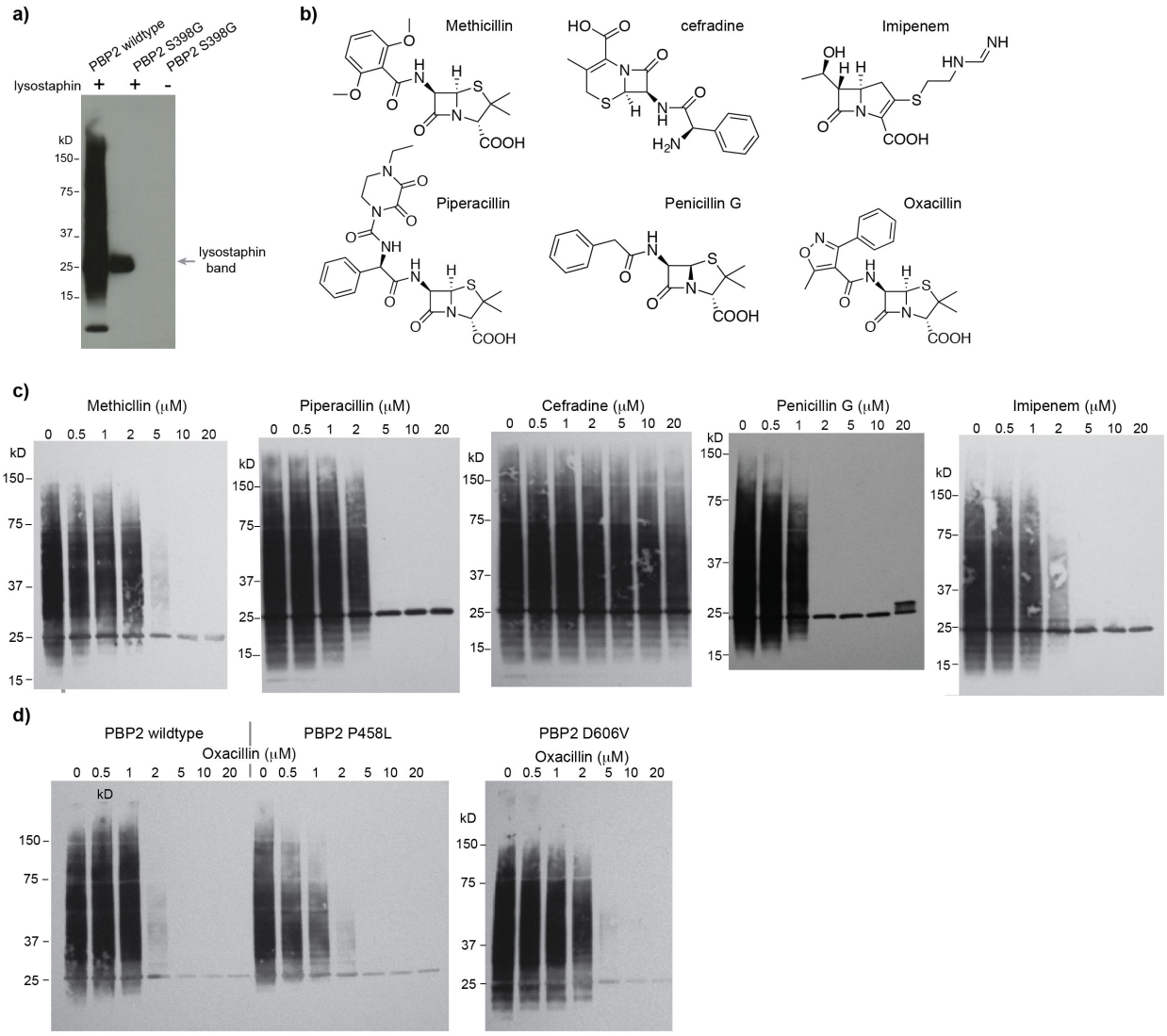


Figure App3.2. Beta-lactams inhibit *S. aureus* PBP2 TP activity *in vitro*. (a) The cross-linking and BDL-labeling are both specific to the TP activity of PBP2. PBP2 S398G (TP inactive mutant) shows no activity in the presence or absence of lysostaphin. (b-c) Structures of beta-lactams and their inhibitory activities of PBP2. (d) PBP2 mutants demonstrate altered TP activities in the presence of specific beta-lactams.