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The First Total Synthesis of Lipid II: The Final Monomeric Intermediate in Bacterial Cell Wall Biosynthesis

Michael S. VanNieuwenhze,* Scott C. Mauldin, Mohammad Zia-Ebrahimi, Brian E. Winger,‡ William J. Hornback, Shankar L. Saha,# James A. Aikins, and Larry C. Blaszczak*

Contribution from Discovery Chemistry Research and the Department of Pharmaceutical and Analytical Chemistry, Lilly Research Laboratories, A Division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285

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Abstract: Bacterial peptidoglycan is composed of a network of β-[1,4]-linked glyan strands that are cross-linked through pendant peptide chains. The final product, the murein sacculus, is a single, covalently closed macromolecule that precisely defines the size and shape of the bacterial cell. The recent increase in bacterial resistance to cell wall active agents has led to a resurgence of activity directed toward improving our understanding of the resistance mechanisms at the molecular level. The biosynthetic enzymes and their natural substrates can be invaluable tools in this endeavor. While modern experimental techniques have led to isolation and purification of the biosynthetic enzymes utilized in peptidoglycan biosynthesis, securing useful quantities of their requisite substrates from natural substrates has remained problematic. In an effort to address this issue, we report the first total synthesis of lipid II (**4**), the final monomeric intermediate utilized by Gram positive bacteria for peptidoglycan biosynthesis.

Introduction

Potent and notably nontoxic cell wall biosynthesis inhibitors have dominated treatment regimens for management of bacterial infections in both hospital and outpatient settings for more than fifty years.[1] Glycopeptide antibiotics (e.g., vancomycin) and β-lactams, two of the most broadly used classes of antibiotics, derive their antibacterial activity through inhibition of key steps in the cell wall biosynthesis cascade.[1] Recently, however, resistance of bacteria to these antibiotics has reached an alarming level[2] and has begun to erode their once dependable clinical efficacy. Augmentation of the cell wall active antibacterial pharmacopeia is urgently needed.

Bacterial peptidoglycan consists of a network of β-[1,4]-linked carbohydrate polymers that are cross-linked via pendant peptide chains.[3] This process is believed to occur in three distinct stages (Scheme 1). The initial stages of the biosynthetic cascade occur within the bacterial cytoplasm and begin with the elaboration of UDP-*N*-acetylglucosamine **1**. First, an enolpyruvyl transferase (MurA) and an NADPH-dependent reductase (MurB) introduce the C(3)-lactyl residue that serves as the anchor for introduction of the pentapeptide side chain. The peptide side chain is then incorporated via sequential addition of L-Ala, D-Glu, L-Lys (or meso DAP[4]), and D-Ala-D-Ala in a series of reactions mediated by ATP-dependent amino acid ligases (MurC-F). The final product of this sequence, UDP-*N*-acetylmuramylpentapeptide (Park nucleotide **2**[5]), then enters the second stage of the bacterial cell wall biosynthetic cascade that takes place at the cytoplasmic surface of the bacterial cell membrane.

In the second stage, a pyrophosphate exchange reaction, catalyzed by MraY, couples Park nucleotide to a membrane-anchored C_{55} lipid carrier. This reaction occurs with concomitant ejection of UMP and provides undecaprenylpyrophosphoryl-MurNAc-pentapeptide **3** (lipid I). Subsequently, MurG catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) fron UDP-GlcNAc to the C(4)-hydroxyl group of the lipid-linked MurNAc-pentapeptide. The product of this transformation, lipid II **4**, is the final monomeric intermediate utilized in bacterial cell wall biosynthesis.[6] In some Gram-positive bacteria, lipid II may be further modified via attachment of 1−5 amino acid residues to the terminal amino group of the lysine residue. These additional residues are frequently glycines, although organisms incorporating L-serine, L-threonine, and other amino acids are known.[2,7]

* Address correspondence to this author. E-mail: msv@lilly.com.
‡ Department of Pharmaceutical and Analytical Chemistry.
# Current address: MediChem Research, Inc., 12305 S. New Avenue, Lemont, IL 60439.

(1) Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. *The Molecular Basis of Antibiotic Action*, 2nd ed.; Wiley-Interscience: New York, 1981.
(2) Review: Chu, D. T. W.; Plattner, J. J.; Katz, L. *J. Med. Chem.* **1996**, *39*, 3853.
(3) (a) Rogers, H. J.; Perkins, H. R.; Ward, J. B. *Biosynthesis of Peptidoglycan*; Chapman and Hall, Ltd.: London, 1980. (b) Höltje, J.-V. *Microbiol. Mol. Bio. Rev.* **1998**, *62*, 181. (c) Bugg, T. D. H.; Walsh, C. T. *Nat. Prod. Rep.* **1992**, 199.
(4) In Gram negative bacteria, *meso*-diaminopimelic acid (*meso*-DAP) is incorporated in place of L-Lys (see ref 3).
(5) Park, J. T. *J. Biol. Chem.* **1952**, *194*, 877.
(6) In Scheme 1, lipid II is shown with a glutamine residue in the pentapeptide side chain replacing glutamate as shown in the lipid I precursor. In some bacterial cells, for example, *Streptococcus pneumoniae*, a glutamine residue is present in lipid II. Experiments have shown lipid I and lipid II (D-Glu) are substrates for an ATP-dependent amidation reaction. See: Siewert, G.; Strominger, J. L. *J. Biol. Chem.* **1968**, *243*, 783.

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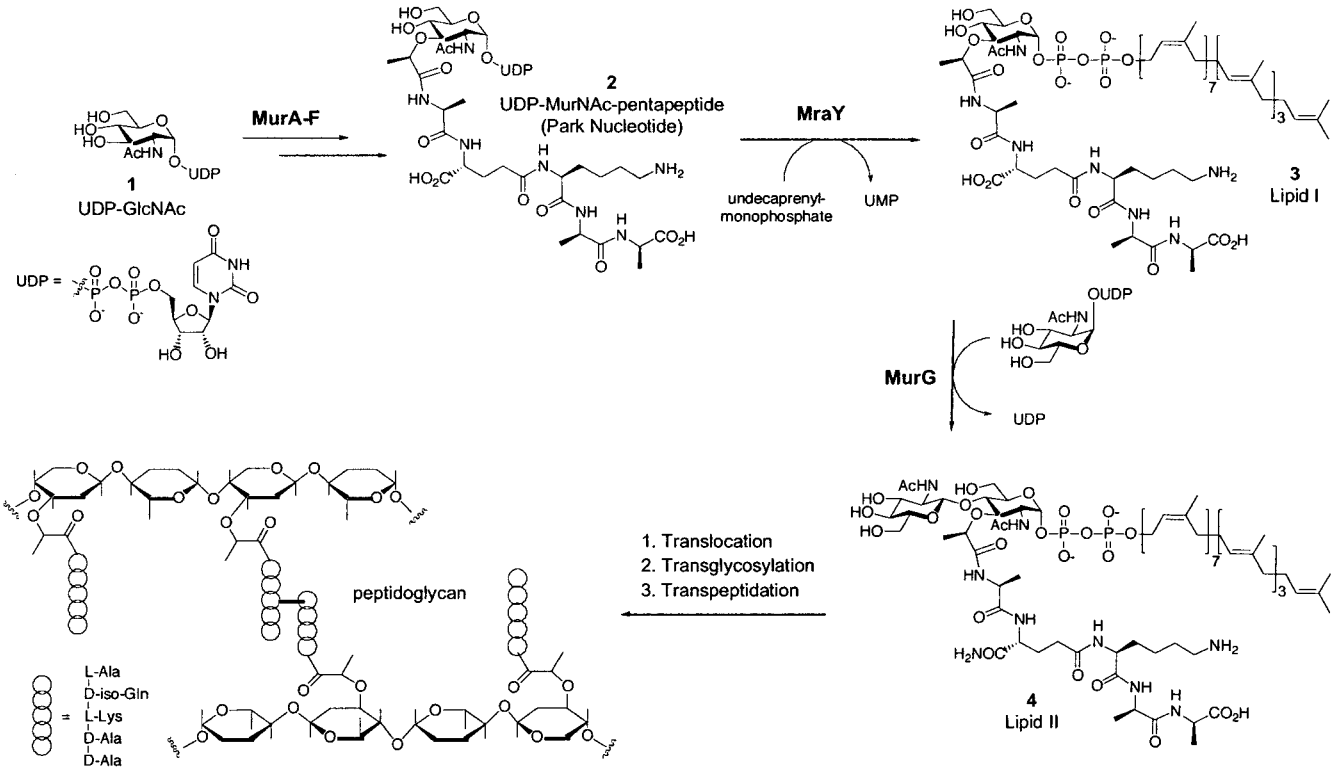
Scheme 1. Biosynthesis of Lipid II and Its Conversion into Peptidoglycan[6]



The last stages of bacterial cell wall biosynthesis are extracytoplasmic. These processes are polymerization of the lipid II intermediate **4** into β-[1,4]-linked glycan strands having an *N*-acetylglucosaminyl-*N*-acetylmuramic acid (NAG-NAM) repeating unit, transpeptidations that extend and cross-link the pendant muramyl peptides of adjacent strands, and carboxypeptidations that modify the peptides. The final product, called the murein sacculus, is a single covalently closed macromolecule that precisely defines the size and shape of a bacterial cell.[3]

Cell wall (peptidoglycan) biosynthetic enzymes and their natural substrates can be invaluable tools in studies directed toward understanding resistance mechanisms at the molecular level, or toward the search for newer and more effective agents. While modern biochemistry and molecular genetics techniques have been used to secure the cell wall biosynthesis enzymes in substantial quantity, acquisition of their respective substrates in useful quantities from natural sources has remained problematic. These substances generally occur in small concentrations within the bacterial cell.[8] In our total synthesis of undecaprenylpyrophosphoryl-MurNAc-pentapeptide (lipid I, **3**),[9,10] we demonstrated that chemical synthesis can provide a powerful solution to this difficult problem.

Our recent studies in peptidoglycan biosynthesis required a supply of lipid II **4**. Isolation of lipid II from bacteria presents several formidable challenges. First, the total pool of **4** present in a bacterial cell culture is very small indeed; the amount has been estimated to be 1000−2000 molecules per cell in *Escherichia coli*.[11,12] Second, complete separation of the miniscule amount of **4** from relatively large amounts of cellular lipid, and membrane, components is tedious at best. Typically, the desired material is followed through purification by scintillation after precursing of the culture with radiolabeled components.[11,13] Isolated yields of **4** are low[14] which, when coupled with the low copy numbers per cell, present difficult technical challenges for its isolation in sufficient quantities to enable detailed mechanistic studies.

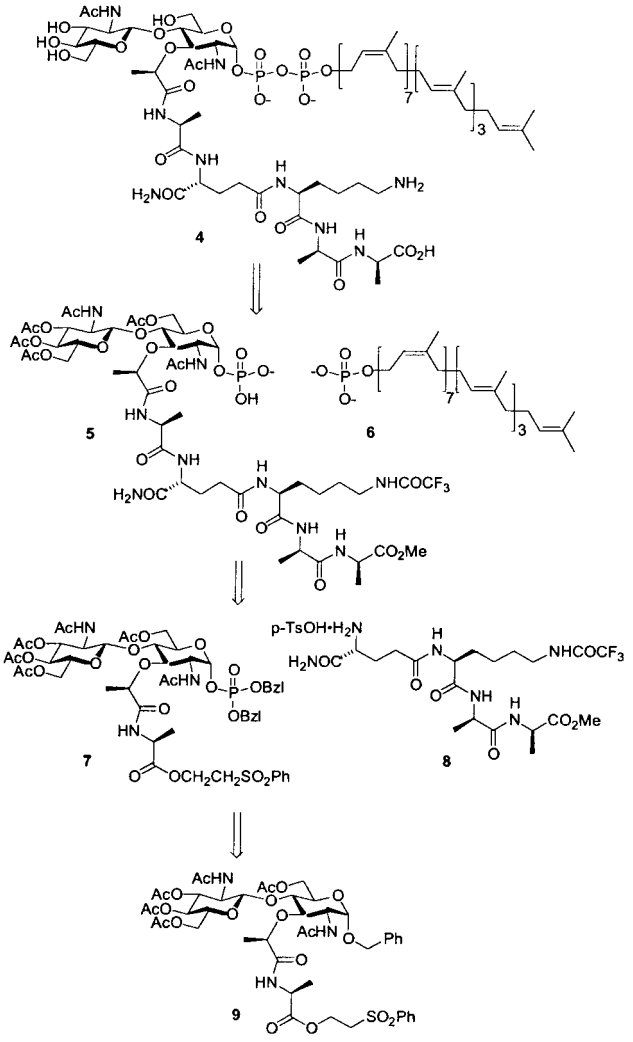
Despite the inherent challenges, we chose to extend our program in the development of practical syntheses of cell wall biosynthetic intermediates to include lipid II. A recent report by Walker disclosed a route to lipid II that utilized MurG and UDP-GlcNAc for the biosynthetic conversion of lipid I into lipid II.[15] We now report the first chemical synthesis of lipid II (**4**), the final monomeric intermediate utilized in bacterial cell wall biosynthesis.[16,17]

Retrosynthetic Analysis

The diversity of structural elements resident in lipid II present several significant challenges for total synthesis. The central

(7) Schleifer, K. H.; Kandler, O. *Bacteriol. Rev.* **1972**, *36*, 407.
(8) Kohlrausch, U.; Höltje, J.-V. *FEMS Microbiol. Lett.* **1991**, *78*, 253 and references therein.
(9) VanNieuwenhze, M. S.; Mauldin, S. C.; Zia-Ebrahimi, M.; Aikins, J. A.; Blaszczak, L. C. *J. Am. Chem. Soc.* **2001**, *123*, 6983.
(10) A total synthesis of UDP-MurNAc-pentapeptide (Park nucleotide) **2** has also been reported by a group at Eli Lilly and Company. See: Hitchcock, S. A.; Eid, C. N.; Aikins, J. A.; Zia-Ebrahimi, M.; Blaszczak, L. C. *J. Am. Chem. Soc.* **1998**, *120*, 1916.
(11) Van Heijenoort, Y.; Gómez, M.; Derrien, M.; Ayala, J.; van Heijenoort, J. *J. Bacteriol.* **1992**, *174* (11), 3549.
(12) Although these data are taken from a gram-negative organism (*E. coli*), the copy numbers are illustrative of the low cellular concentration of lipid II.
(13) See, for example: (a) Umbreit, J. N.; Strominger, J. L. *J. Bacteriol.* **1972**, *112*, 1306. (b) Nakagawa, J.; Tamaki, S.; Tomioka, S.; Matsuhashi, M. *J. Biol. Chem.* **1984**, *259*, 13937. (c) Brötz, H.; Bierbaum, G.; Leopold, K.; Reynolds, P. E.; Sahl, H.-J. *Antimicrob. Agents Chemother.* **1998**, *42*, 154.
(14) For example, Umbreit and Strominger reported yields of 2% (ref 12a). A cell-free system (ref 11) has been reported that has provided improved yields of lipid II (12−18%).
(15) Ye, X.-Y.; Lo, M.-C.; Brunner, L.; Walker, D.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2001**, *123*, 3155.

Scheme 2. Retrosynthetic Analysis for Lipid II



core of lipid II consists of a β-[1,4]-linked disaccharide containing *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) subunits. The muramyl residue is further decorated with a pentapeptide chain (L-Ala-D-*i*-Gln-L-Lys-D-Ala-D-Ala) attached to the 3-position via a lactyl linkage, as well as a chemically sensitive undecaprenyl-linked α-glycosyl diphosphate moiety. Our retrosynthetic analysis for the construction of lipid II is illustrated in Scheme 2.

First, we felt it would be optimal to introduce the undecaprenyl-linked diphosphate moiety at a late stage in the synthesis. This would allow us to avoid potential solubility complications in subsequent reactions that could arise from the enhanced lipophilic character of the undecaprenyl-linked substrate. In addition, late stage introduction of the lipid-linked diphosphate would minimize the number of subsequent synthetic operations this chemically sensitive allylic diphosphate linkage must withstand.

Second, the anticipated acid sensitivity of the anomeric diphosphate dictated the use of base-cleavable protective groups for all functional groups that are unmasked after its installation. As a result, acetate was chosen to block the hydroxyl groups of the disaccharide core, trifluoroacetate was chosen to mask the ϵ-amino group of L-lysine, and methyl ester was chosen to protect the carboxylic acid moiety of the terminal D-alanine residue. Global deprotection could then be accomplished by exposure to hydroxide ion as the final step in the synthesis.[18]

In analogy to our lipid I total synthesis,[9] our initial disconnection reveals disaccharyl pentapeptide **5** and undecaprenyl monophosphate **6**.[19] Disaccharyl pentapeptide **5** was envisaged to derive from coupling of a suitably protected tetrapeptide fragment (e.g., **8**) with an activated ester deriving from **7**.[20] The tetrapeptide fragment **8** would be prepared incorporating base-cleavable protective groups for the ϵ-amino group of the L-lysine residue and carboxy terminal D-alanine residue. An important consideration here, with respect to synthetic design of **7**, is that the protecting groups for the L-Ala residue and the anomeric phosphate are orthogonal to one another as are those protective groups (acetates) used for peripheral hydoxyl groups of the disaccharide core. Triple orthogonality is required to ensure selective unmasking of functional groups prior to coupling of the tetrapeptide fragment, and installation of the undecaprenyl side chain.

We envisaged a phosphitylation/oxidation[21,22] sequence for stereoselective introduction of the anomeric phosphate in **7**. Thus, **7** could be derived from disaccharyl monopeptide **9** where, again, a triply orthogonal protection scheme would allow selective unmasking of the anomeric hydroxyl group prior to introduction of the anomeric phosphate.

To initiate our synthesis of lipid II, an efficient synthetic route to multiple gram quantities of orthogonally protected *N*-acetyl-(2-deoxy-2-aminoglucopyranosyl)-β-[1,4]-*N*-acetylmuramyl (NAG-NAM) monopeptide **9** was desired. Access to **9** would require a reliable method for establishing the desired β-[1,4]-glycosidic bond and a functional group activation/protection strategy that would meet the demands of triple orthogonality. Several synthetic approaches to protected versions of the NAG-NAM disaccharide subunit have been recorded previously.[23] Our synthetic route, which employed a triply orthogonal protection scheme, readily provided NAG-NAM monopeptide **9** in multiple gram quantities.[24]

The Total Synthesis of Lipid II

Our synthesis (Scheme 3) began with hydrogenolysis of benzyl ether **9** to provide lactol **10** in 94% yield. The stage was now set for introduction of the anomeric phosphate group. As

(16) (a) VanNieuwenhze, M. S. The First Total Synthesis of Lipid II: The Final Monomeric Intermediate in Bacterial Cell Wall Biosynthesis; Presented at the Gordon Research Conference on Stereochemistry, Newport, RI, 2000. (b) VanNieuwenhze, M. S.; Mauldin, S. C.; Zia-Ebrahimi, M.; Hornback, W. J.; Saha, S. L.; Blaszczak, L. C. *Abstracts of the 221st National Meeting of the American Chemical Society, San Diego, California, April 2001*; American Chemical Society: Washington, DC, 2001; Abstract No. 470. (c) Blaszczak, L. C.; VanNieuwenhze, M. S.; Zia-Ebrahimi, M.; Mauldin, S. C.; Skatrud, P. L.; Alborn, W. E. PCT WO 01/79268, filed April 5, 2001.

(17) Scientists at DuPont Pharmaceuticals have recently published a synthesis of lipid II. Schwartz, B.; Markwalder, J. A.; Wang, Y. *J. Am. Chem. Soc.* **2001**, *123*, 11638.

(18) Care would have to be exercised when exposing the pentapeptide to hydroxide ion-mediated deprotection conditions since the base-catalyzed rearrangement of iso-glutamine to glutamate in peptidoglycan-related structures has been observed. (a) Keglevic, D.; Derome, A. E. *Carbohydr. Res.* **1989**, *186*, 63. (b) Keglevic, D.; Kidric, J. *J. Carbohydr. Res.* **1992**, *11*, 119.

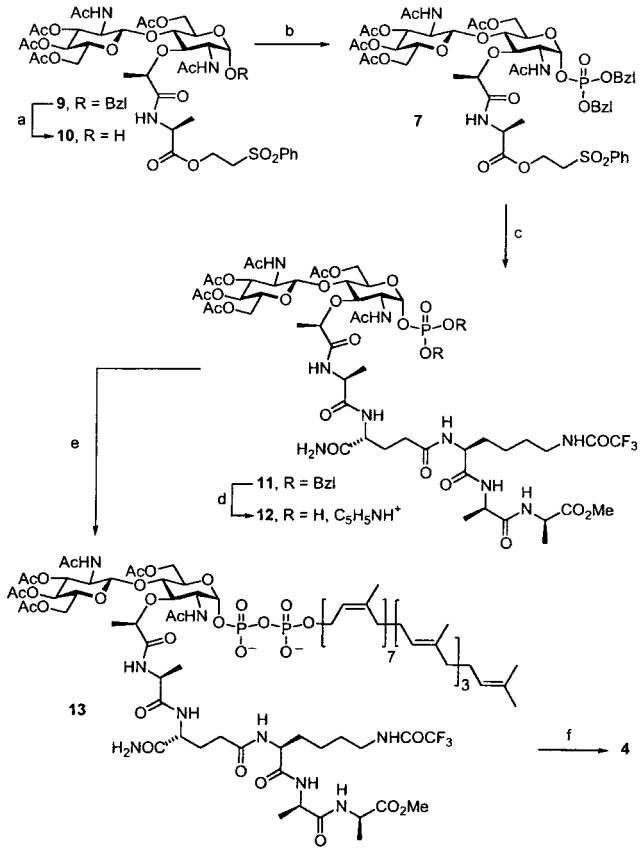
(19) Undecaprenyl monophosphate bis-ammonium salt may be purchased from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5A, 02-106 Warszawa, Poland.

(20) The rationale for installing the peptide side chain as a tetrapeptide versus a pentapeptide is an artifact of the synthetic strategy used for preparation of **6**. For a more detailed discussion, please refer to ref 24.

(21) Sim, M. M.; H. Kondo, H.; Wong, C.-H. *J. Am. Chem. Soc.* **1993**, *115*, 2261.

(22) Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1998**, *120*, 2484.

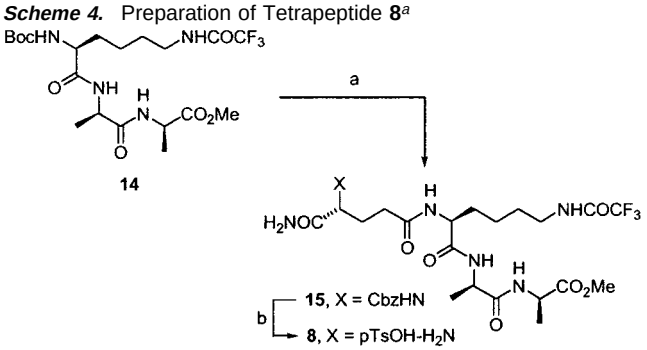
Scheme 3. The Total Synthesis of Lipid II[a]



[a] Reagents and conditions: (a) H_2, Pd/C, MeOH/THF, 94% yield; (b) *i*. dibenzyl-*N*,*N*-diethylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2; *ii*. 30% H_2O_2, THF, −78 °C to room temperature, 78% yield; (c) *i*. DBU, CH_2Cl_2; *ii*. EDCI, NHS, DMF, then **8** and $^iPr_2NEt$, 46% yield; (d) H_2, Pd/C, MeOH, then pyridine, 91% yield; (e*) i.* CDI/DMF/THF; *ii*. undecaprenyl monophosphate **6** (bis-NH_4^+ salt); (f) NaOH/H_2O/1,4-dioxane, 24% overall yield from **11**.

was the case in our lipid I synthesis, the phosphate group needed to be introduced in an α-selective fashion. Given this requirement, and the presence of a participating group (NHAc) at C(2), a sequence employing a nucleophilic carbohydrate component and an electrophilic phosophorus reagent was mandated.[25] As a result, our initial efforts focused on the phosphitylation/oxidation sequence that was exploited during our total synthesis of lipid I. Even with this precedent, we were still concerned about the possible erosion of the anomeric selectivity that would arise if in situ anomerization of the free hydroxyl group occurs at a rate that is competitive with capture by the activated phosphorus electrophile. This could become problematic if the steric constraints imposed by the additional sugar subunit compromised the nucleophilicity of the anomeric hydroxyl group.

Scheme 4. Preparation of Tetrapeptide **8**[a]



[a] Reagents and conditions: (a) *i*. CH_2Cl_2, TFA; *ii*. Boc-D-*iso*-Gln(NHS), $^iPr_2NEt$, THF, 66% yield; (b) EtOH/CH_2Cl_2, *p*-TsOH, 97% yield.

We were gratified to observe that, after exposure of lactol **10** to dibenzyl-*N*,*N*-diethylphosphoramidite and 1*H*-tetrazole in dichloromethane followed by oxidation of the phosphite intermediate with 30% hydrogen peroxide, the desired α-phosphate product **7** was obtained in 78% yield ($^3J_{H1H2}$ = 3.0 Hz). Our next step set the stage for elaboration of the pentapeptide side chain. Thus, unmasking of the lactyl carboxyl group was achieved through treatment of phenylsulfonylethyl ester **7** with DBU. The intermediate acid was activated through conversion to the corresponding NHS ester. Addition of a DMF solution of tetrapeptide **8**, prepared via standard peptide synthesis protocols (Scheme 4[26]), to a solution of the activated ester and $^iPr_2NEt$ in DMF provided disaccharyl pentapeptide **11** (46% overall yield from **7**).[27]

Hydrogenolytic cleavage of the phosphodiester protecting groups, followed by evaporation of the crude product from pyridine, provided monopyridyl salt **12** in 91% yield. The stage was now set for the final lipid coupling/deprotection sequence that would allow completion of our lipid II total synthesis.

For the key reaction that would establish the lipid diphosphate linkage, we utilized the phosphoroimidazolidate method[28] that was exploited in the lipid I total synthesis. The attractive feature of this method is that it would allow us to employ commercially available undecaprenyl monophosphate directly in a coupling reaction with a carbohydrate-derived phosphoroimidazolidate. The mild reaction conditions were also advantageous for introduction of the chemically sensitive diphosphate linkage. In the event, electrophilic activation, achieved by in situ conversion to the intermediate phosphoroimidazolidate, followed by exposure to undecaprenyl monophosphate **6** (bis-NH_4^+ salt) in DMF/THF over 4 days cleanly afforded the fully protected version of lipid II **13**.[29] Global deprotection was achieved through exposure of **13** to aqueous NaOH and provided lipid II **4** in 24% isolated yield (from **11**) after reverse-phase HPLC purification.[30]

(23) For previous approaches to the β-[1,4]-linked NAG-NAM disaccharide, see: (a) Merser, C.; Sinay, P. *Tetrahedron Lett.* **1973**, *13*, 1029. (b) Durette, P. L.; Meitzner, E. P.; Shen, T. Y. *Carbohydr. Res.* **1979**, *77*, C1. (c) Kiso, M.; Kaneda, Y.; Shimizu, R.; Hasegawa, A. *Carbohydr. Res.* **1980**, *83*, C8. (d) Kiso, M.; Kaneda, Y.; Shimizu, R.; Hasegawa, A. *Carbohydr. Res.* **1982**, *104*, 253. (e) Kusumoto, S.; Yamamoto, K.; Imoto, M.; Inage, M.; Tsujimoto, M.; Kotani, S.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 1411. (f) Kusimoto, S.; Imoto, M.; Ogiku, T.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 1419. (g) Farkas, J.; Ledvina, M.; Brokes, J.; Jezek, J.; Zajicek, J.; Zaoral, M. *Carbohydr. Res.* **1987**, *163*, 63. (h) Kinzy, W.; Schmidt, R. R. *Liebigs Ann. Chem.* **1987**, 407. (i) Kantoci, D.; Keglevic, D.; Derome, A. *Carbohydr. Res.* **1987**, *162*, 227. (j) Termin, A.; Schmidt, R. R. *Liebigs Ann. Chem.* **1989**, 789. (k) Ledvina, M.; Farkas, J.; Zajicek, J.; Jezek, J.; Zaoral, M. *Collect. Czech. Chem. Commun.* **1989**, *54*, 2784. (l) Termin, A.; Schmidt, R. R. *Liebigs Ann. Chem.* **1992**, 527.

(24) Saha, S. L.; VanNieuwenhze, M. S.; Hornback, W. J.; Aikins, J. A.; Blaszczak, L. C. *Org. Lett.* **2001**, 3, 3575.

(25) Carbohydrates bearing functional groups at C(2) capable of neighboring group participation generally favor formation of 1,2-*trans*-linked glycosyl phosphates.

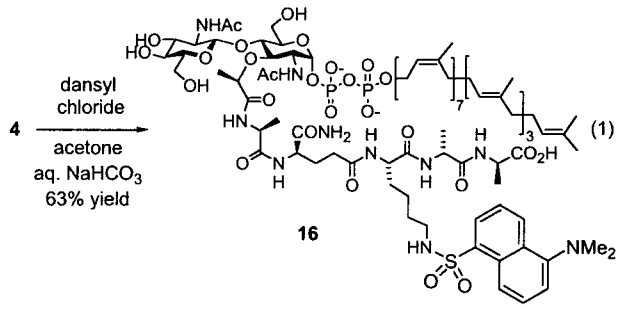
(26) Tripeptide **14** was prepared as described in ref 10.

(27) A small amount (<5%) of a second product, of identical molecular weight, is routinely observed during the coupling reaction. This product may be a diastereomer that could, presumably, arise from epimerization of the L-Ala α-stereocenter during the peptide-coupling event.

(28) (a) Fang, X.; Gibbs, B. S.; Coward, J. K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2701. (b) Danilov, L. L.; Maltsev, S. D.; Shibaev, V. N.; Kochetkov, N. K. *Carbohydr. Res.* **1981**, *88*, 203.

(29) Undecaprenyl monophosphate was added in portions and reaction progress was monitored, through tracking the disappearance of the intermediate phosphoroimidazolidate, by mass spectroscopy.

The structural identity of lipid II was further validated via a modification of a known biochemical assay[31] that employed dansylated derivative **16** in place of radiolabeled lipid II of



bacterial origin. Weppner and Neuhaus had previously demonstrated that Park nucleotide bearing a dansyl tag on the L-lysine residue (e.g., a dansyl-tagged version of **2**) could be incorporated into glycan strands upon incubation with a membrane fraction prepared from *Gaffkya homari*.[32] In the event, formation of glycan strands via enzymatic polymerization of **16** was readily confirmed by inspection of descending paper or thin layer (silica gel) chromatograms upon exposure to ultraviolet light.[32,33]

(30) Please refer to the Supporting Information for experimental details.
(31) Suzuki H.; van Heijenoort, Y.; Tamura, T.; Mizoguchi, J.; Hirota, Y.; van Heijenoort, J. *FEBS Lett.* **1980**, *110(2)*, 245.

New therapeutic agents capable of disrupting steps in bacterial cell wall biosynthesis, yet to be exploited by existing agents, offer one potential strategy in the continuing battle against antimicrobial resistance. Heretofore, detailed study of resistance mechanisms at the molecular level, despite the ready availability of the biosynthetic enzymes, has lagged due to the limited availability of the natural substrates. This synthesis of lipid II provides a valuable biochemical tool that may prove useful in uncovering novel approaches to combat emerging antimicrobial drug resistance to cell wall active agents.

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Supporting Information Available: Complete experimental procedures and spectral data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA017386D

(32) Weppner, W. A.; Neuhaus, F. C. *J. Biol. Chem.* **1977**, *252*(7), 2296.
(33) A similar experiment utilizing a membrane particulate fraction from *E. coli* with a dansyl-tagged version of lipid I (the immediate biosynthetic precursor to lipid II) has also been reported. See: Auger, G.; Crouvoisier, M.; Caroff, M.; van Heijenoort, J.; Blanot, D. *Lett. Pept. Sci.* **1997**, *4*, 371.