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Synthesis of P^1-Citronellyl-P^2-α-D-pyranosyl Pyrophosphates as Potential Substrates for the *E. coli* Undecaprenyl-pyrophosphoryl-*N*-acetylglucoseaminyl Transferase MurG

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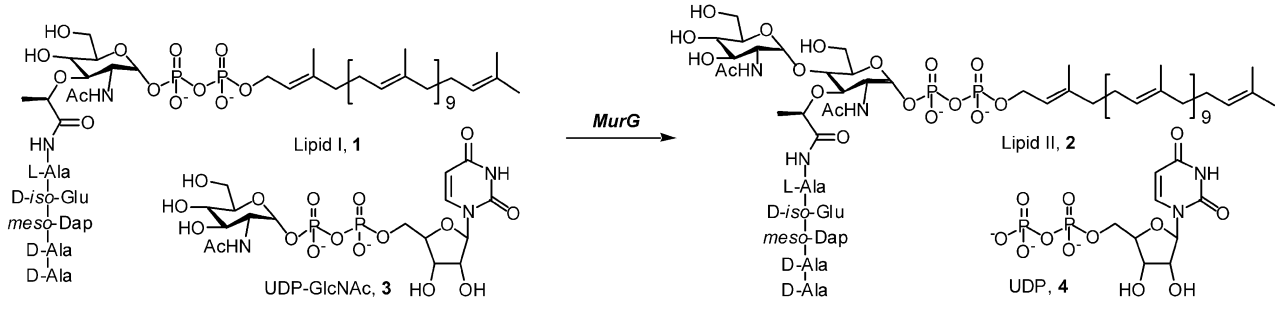
Abstract—P^1-Citronellyl-P^2-α-D-pyranosyl pyrophosphates containing α-D-*N*-acetylglucoseaminyl, α-D-glucosyl, and α-D-*N*-acetylmuramyl carbohydrates were synthesized and used in substrate specificity studies of the *Escherichia coli* MurG enzyme. Oxalyl chloride activation of citronellyl phosphate for coupling to α-D-pyranose-1-phosphates resulted in markedly improved yields over traditional Khorana–Moffatt and diphenyl chlorophosphate activation strategies.

In bacterial cell-wall biosynthesis, the enzyme MurG catalyzes the final membrane-associated step in the biosynthesis of the peptidoglycan monomer.[1–3] MurG utilizes the substrates uridyl-diphospho-*N*-acetylglucosamine (UDP-GlcNAc, **3**) and undecaprenyl-pyrophosphoryl-*N*-acetyl-muramyl-pentapeptide (Lipid I, **1**) to produce uridyl-diphosphate (UDP, **4**) and undecaprenyl - pyrophosphoryl - *N* - acetyl - muramyl - pentapeptide (Lipid II, **2**) (Scheme 1). Lipid II (**2**) is subsequently exported to the outer surface of the bacterial cell where it becomes polymerized and crosslinked into mature peptidoglycan by transglycosylases. Because MurG catalyzes an essential cell wall biosynthesis step, it is emerging as an attractive target for the development of new antimicrobial agents to help stave off infections due to multidrug resistant microorganisms.

For years, a limiting factor barring analysis of MurG has been its difficult purification away from the membrane and the lack of availability of its natural substrate, Lipid I (**1**), due to its low natural abundance,[4] chemical complexity, difficult isolation,[5] and propensity to form detergent-like micelles.[4] Recent advances in the cloning,[6] overexpression,[1,7,8] purification,[7,8] and crystal structure[9] of *Escherichia coli* MurG together with the discovery of water-soluble substrates[8,10] and novel assay



Scheme 1. The bacterial peptidoglycan biosynthesis MurG reaction.

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methods[8,10,11] have paved the way for detailed analysis of its mechanism and substrate specificity. Van Heijenoort and co-workers[12] prepared a fluorescent analogue of Lipid I, heptaprenyl-pyrophosphoryl-MurNAc-pentapeptide (N^{ε}-Lys-dansylated) from naturally-isolated UDP-*N*-acetylmuramyl pentapeptide[13] (Park's nucleotide). Hitchcock and co-workers[14] of Lilly Research laboratories recently developed a convergent total synthetic route to Park's nucleotide from *O*-benzyl-*N*-acetyl-4,6-benzylidinemuramic acid,[15] providing the first route to this class of compounds via total synthesis, thus facilitating synthetic modification of the peptidoglycan intermediate architecture. Building upon this precedent, VanNieuwenhze and colleagues[16] (also from Lilly) recently reported the first total synthesis of native undecaprenyl–Lipid I. In a related approach, Walker and co-workers[10] synthesized an analogue of Lipid I (**5**) with two modifications that improved solubility and facilitated affinity capture and assay detection. Walker's group replaced the C-55 undecaprenol native lipid with a shorter C-10 chain derived from (*R*)-(+)-β-citronellol and also labeled the molecule with D-biotin.

Preliminary substrate specificity studies with soluble preparations of MurG have shown a distinct preference for the authentic Lipid I substrate, and a capacity for glycosylating smaller, truncated Lipid I analogues such as **6**, albeit with less efficiency. This observation has introduced the possibility of using smaller, synthetically tractable surrogate substrates for mechanistic evaluation or inhibitor screening. Since the multistep synthesis of **5** is costly and time consuming,[8,10] we set out to both improve key elements of this synthesis that were not amenable to preparative scaleup and to also establish if simpler P^1-citronellyl-P^2-α-D-pyranosyl pyrophosphates containing α-D-*N*-acetylglucoseaminyl, α-D-glucosyl, and α-D-*N*-acetylmuramyl carbohydrates might be kinetically competent minimal substrate replacements. We report here a new synthesis of full-length citronellyl–Lipid I and three related P^1-citronellyl-P^2-α-D-pyranosyl pyrophosphates and their evaluation as potential substrates for MurG.

P^1-Citronellyl-P^2-α-D-glucosyl pyrophosphate (**14**) and the related P^1-citronellyl-P^2-α-D-*N*-acetylglucoseaminyl pyrophosphate (**15**) were prepared in six steps from hyperacetylated carbohydrate precursors **8** and **9**, respectively (Scheme 2). Selective deacetylation of C-1 with hydrazine acetate[17] (69–74% yield) followed by four step conversion of the anomeric alcohol provided the glycosyl phosphates **10** and **11** in 42 and 35% yields, respectively. (*R*)-(+)-β-citronellyl phosphate (**7**) was prepared separately according to the method of Danilov and Chojancki.[18] Attempts at Khorana–Moffatt[18] activation of **7** for pyrophosphate bond formation produced almost exclusively the guanidine byproduct, normally an undesirable side reaction associated with this procedure.[19] Similarly, use of diphenyl chlorophosphate[20] for activation of **7** resulted in erradic yields of the desired pyrophosphate.

The solution to this problem was found by using oxalyl chloride as an activator to form the highly reactive dichlorophosphate of **7**.[21] Treatment of **7** with 20 equiv of oxalyl chloride and glycosyl phosphates **10** and **11** rapidly generated the desired pyrophosphates **12** and **13** in good yields after hydrolytic workup with acetone/H_2O/triethylamine (88:10:2, v/v/v; 2 h, rt) (75 and 70% yields, respectively).[21] Lastly, to facilitate complete deprotection of the acetates, 2.2 equiv of sodium methoxide in methanol were required to provide compounds **14** and **15** in 70 and 65% yields, respectively. All intermediates and final products were characterized by NMR and ESI-MS.[22,23]

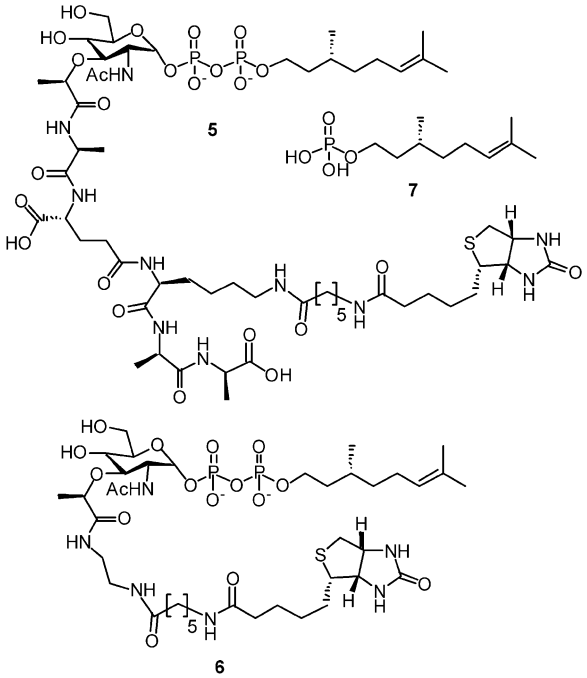
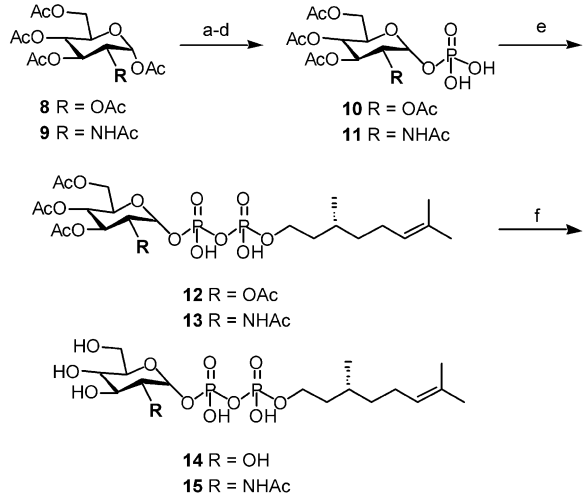


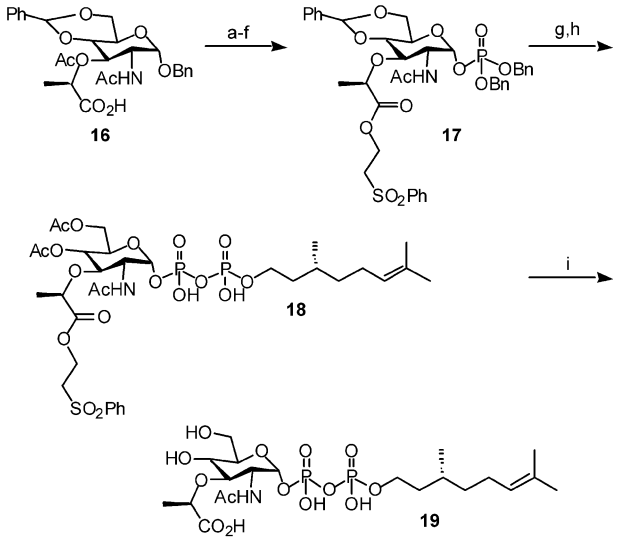
Figure 1. Biotinylated citronellyl–Lipid I (**5**) and two related fragments.



Scheme 2. Reactions and conditions: (a) H_2NNH_2 acetate, DMF, 15 min, 50 °C; (b) dibenzyl *N,N*-diethylphosphoramidite, 1,2,4-triazole, CH_2Cl_2, rt; (c) 30% H_2O_2, THF, −70 °C to rt; (d) H_2, Pd/C, cyclohexylamine, MeOH, 16 h; (e) (*R*)-(+)-β-citronellyl-$OPOCl_2$, pyridine, DMF, 1.5 h, rt; (f) NaOMe 2.2 equiv, MeOH, 15 min, rt.

P^1-Citronellyl-P^2-α-D-*N*-acetylmuramyl pyrophosphate (**19**) was synthesized from *O*-benzyl 4,6-benzylidine-muramic acid[15] (**16**). First, benzylidine **16** was converted to dibenzylphosphate **17** in 30% yield over six steps according to the method of Hitchcock and colleagues.[14] Hydrogenolysis of **17** followed by oxalyl chloride-mediated coupling to **7** produced pyrophosphate **18** in 74% yield over two steps. Lastly, global deacetylation with NaOMe/MeOH produced the desired compound **19** in 78% yield. Compound **19** and intermediates were characterized by elemental analysis, NMR and ESI-MS (Scheme 3).[24]

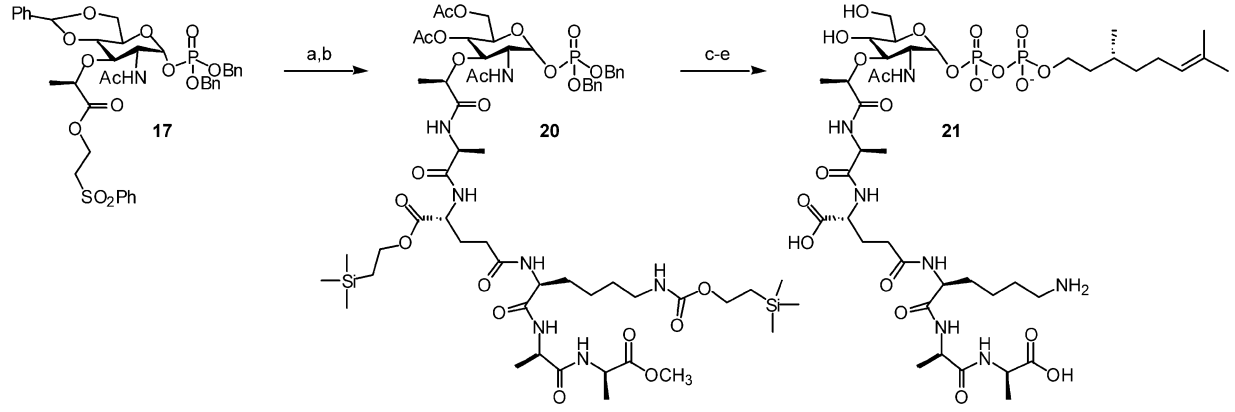
Walker's total synthesis of citronellyl–Lipid I (**5**) on a ~1.0 mmol scale[8] employed diphenyl chlorophosphate activation of **7** for assembly of the key pyrophosphate bond. Although this reaction occurred in 68% yield, during preparative scale up of this synthesis (>25 mmol), we routinely observed a marked reduction in yields for this step (<35% yield). To address this issue, a preparative synthesis of citronellyl-Lipid I was devised whereby oxalyl chloride activation was successfully employed as a replacement for the diphenyl chlorophosphate method (Scheme 4).



Scheme 3. Reactions and conditions: (a) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 2-(phenylsulphonyl)ethanol, DMAP, CH_2Cl_2, 0 °C to rt, 2 h, 96%; (b) AcOH, H_2O, reflux, 45 min; (c) Ac_2O, pyridine, 0 °C to rt, 85%; (d) H_2, Pd/C, AcOH, 16 h, 90%; (e) dibenzyl *N,N*-diethylphosphoramidite, 1,2,4-triazole, CH_2Cl_2, rt; (f) 30% H_2O_2, THF, −70 °C to rt, 32%; (g) H_2, Pd/C, cyclohexylamine, MeOH, 16 h, quantitative; (h) (*R*)-(+)-β-citronellyl-$OPOCl_2$, pyridine, DMF, 1.5 h, rt, 74%; (i) NaOMe 2.2 equiv, MeOH, 15 min, rt, 78%.

On a 25 mmol scale, the phenylsulfonylethyl ester of protected phosphate **17**[14] was removed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene(1,5-5) (DBU) and the resulting acid was coupled to H-L-Ala-D-*iso*-Glu(OTMSE)-L-Lys(TEOC)-D-Ala-D-Ala-OMe[25] using PyBOP, HOBt, and NMM to yield compound **20** in 15% overall yield over 10 steps.[26] Peptide **20** was quantitatively deprotected by catalytic hydrogenation and subsequently coupled to **7** using oxalyl chloride activation (74% yield after hydrolytic workup with acetone/H_2O/triethylamine solvent mixture (88:10:2 v/v/v) for 2 h at rt). Global deprotection was accomplished by treatment with NaOMe/MeOH followed by tetrabutylammonium fluoride (TBAF). Preparative reversed-phase HPLC purification over octadecyl silica (Vydac C_{18}, 50 mM ammonium acetate, pH 4.5) afforded pure **21** in 5% overall yield from **16**. NMR and ESI-MS spectra for **21** exactly matched that of reported values.[10]

To evaluate the activity of compounds **14**, **15**, **19**, and **21**, a new activity assay for MurG was devised. Instead of monitoring the production of Lipid II, this discontinuous assay monitored the conversion of the second MurG substrate, UDP-GlcNAc to UDP using thin-layer chromatography. [α-^{32}P]-UDP-GlcNAc was prepared chemo-enzymatically from glucoseamine-1-phosphate and [α-^{32}P]-UTP according to the method of Anderson and co-workers.[27] Soluble recombinant *E. coli* MurG was prepared according to the method of Walker.[8] Conversion of [α-^{32}P]-UDP-GlcNAc to [α-^{32}P]-UDP was monitored by TLC on PEI-cellulose plates that were eluted with 0.3 M guanidine hydrochloride in water and visualized by phosphorimagery (Molecular Dynamics). Compounds **21**, **14**, **15**, or **19** (100 μM) and [α-^{32}P]-UDP-GlcNAc (100 μM) were incubated with MurG (0.01 μg/μL) for 20 min at 25 °C in 20 mM HEPES pH 8, 2 mM $MgCl_2$. Upon



Scheme 4. Reactions and conditions: (a) DBU, CH_2Cl_2, rt; (b) PyBOP, NMM, L-Ala-γ-D-Glu(O*T*mse)-L-Lys(Teoc)-D-Ala-D-Ala(OMe), DMF, rt; 65% over two steps; (c) H_2, Pd/C, cyclohexylamine, MeOH, 16 h, quant; (d) (*R*)-(+)-β-citronellyl-$OPOCl_2$, pyridine, DMF, 1.5 h, rt, 74%; (e) NaOMe 2.2 equiv, MeOH, 15 min, rt, 46%.

completion of the reaction, 5 μL of the reaction mixture was spotted onto the TLC plates and the plates were eluted with 0.3 M guanidine hydrochloride ([α-^{32}P]-UDP-GlcNAc R_f 0.77; [α-^{32}P]-UDP R_f 0.11). Though full-length citronellyl-Lipid I **21** was processed by the enzyme, the truncated pyrophosphates were not recognized as substrates by soluble MurG to the sensitivity limits afforded by this assay (<0.01% product conversion). Increasing the concentration of compounds **14**, **15**, and **19** (100-fold), the concentration of MurG (100-fold), and the time of the reaction (120 min) did not improve turnover of these compounds. Interestingly, in *trans* addition of free L-Ala-D-*iso*Glu-L-Lys-D-Ala-D-Ala pentapeptide (Bachem) also had no stimulatory effect on the MurG reaction with compounds **14**, **15**, and **19**. Neither compounds **14**, **15**, or **19** possessed antimicrobial activity against a *Bacillus subtilis* Gram-positive bacterial strain.

This data, taken together with prior substrate specificity studies conducted with soluble and membrane preparations of the enzyme[8,28] suggests that MurG requires P^1-lipid-P^2-*N*-acetylmuramyl pyrophosphate substrates containing an *intact* amide between the muramyl lactyl ether sidechain and the pentapeptide. The length and composition of this peptide is important, for truncation of this pentapeptide to diamide **6** reduced activity by 500-fold as compared to **5**,[8] and elimination of the lactate amide substituent in the related compound **19** eliminated activity altogether. In an effort to elucidate the molecular basis of MurG specificity, further structure–activity studies with synthetic P^1-lipid-P^2-α-D-pyranosyl pyrophosphate peptides are underway and will be reported in due course.

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22. Analytical data for **14**: MS (ESI) *m*/*z* 477.5 (M−H); ^{1}H NMR (D_2O, 250 MHz) δ 0.87 (d, J=7.3 Hz, 3H), 1.17–1.51 (m, 7H), 1.60 (s, 3H), 1.66 (s, 3H), 2.01 (m, 2H) 3.31–4.6 (m, 6H), 5.00 (s, 1H), 5.22 (s, 1H).
23. Analytical data for **15**: MS (ESI) *m*/*z* 520.5 (M+H); ^{1}H NMR (D_2O, 250 MHz) δ 0.89 (s, 3H), 1.05–1.35 (m, 7H), 1.61 (s, 3H), 1.67 (s, 3H), 1.81 (s, 3H), 2.06 (m, 3H), 3.66–3.97 (m, 6H), 5.22 (s, 1H), 5.48 (s, 1H).
24. Analytical data for **19**: MS (ESI) *m*/*z* 748.6 (M+H), ^{1}H NMR (CD_3OD, 250 MHz) δ 0.95 (d, J=6 Hz, 3H), 1.38 (d, J=6.7 Hz, 3H), 1.42 (m, 1H), 1.59 (s, 3H), 1.66 (s, 3H), 1.80 (s, 3H), 1.88 (s 1H), 2.08 (m, 2H), 3.20–3.90 (m, 7H), 4.60 (m, 4H), 5.78 (m, 2H); ^{13}C NMR (DMSO-d_6, 500 MHz) 19.93, 19.97, 22.00, 22.51, 54.00, 55.66, 60.29, 69.55, 70.71, 77.57, 77.92, 78.91, 80.69, 94.55, 127.40, 171.72, 174.97.
25. The protected pentapeptide was prepared from Cbz-D-Ala-D-Ala-OMe in 65% overall yield using standard solution-phase methods (PyBOP, HOBt, NMM) and Cbz amino acids.
26. Abbreviations: PyBOP, (benzotriazol-1-yloxy)tris-pyrrolidinophosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine.
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