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# The Kinetic Characterization of *Escherichia coli* MurG Using Synthetic Substrate Analogues

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**Abstract:** Bacterial resistance to existing antibiotics poses a serious threat to human health. Because the peptidoglycan layer surrounding bacterial cells is essential for survival, the enzymes involved in peptidoglycan biosynthesis are attractive targets for the design of new antibiotics. Unfortunately, many of these enzymes are difficult to study because substrates to monitor enzymatic activity are either not available or not soluble under suitable assay conditions. These problems can be solved by utilizing synthetic alternative substrates. We recently reported the synthesis of a soluble substrate analogue for MurG, the enzyme that forms the $\beta$-(1,4)-*N*-acetylglucosaminyl-*N*-acetylmuramyl pentapeptide subunit of peptidoglycan. Using this substrate analogue, we have been able to develop a direct assay to monitor the activity of the enzyme. We now report the purification of *Escherichia coli* MurG and information on its kinetic properties and substrate requirements in the absence of membranes. This work lays the foundation for detailed mechanistic and structural investigations of this essential bacterial enzyme.

## Introduction

Bacterial cell membranes are surrounded by layers of peptidoglycan, a rigid mesh of $\beta$-1,4-linked carbohydrate polymers cross-linked by peptide chains.[1] The peptidoglycan layers protect bacterial cells from lysing under high internal osmotic pressures. Many of the best antibiotics function by inhibiting peptidoglycan biosynthesis. Because resistance to these and other antibiotics is increasing rapidly,[2−5] there has been a resurgence of interest in studying the enzymes involved in peptidoglycan biosynthesis as well as other metabolic pathways in bacteria. It is hoped that structural and mechanistic information on essential bacterial enzymes will facilitate the rational design of new antibiotics.

Peptidoglycan biosynthesis takes place in three distinct stages[6,7] (Scheme 1). Stage I occurs in the cytosol and involves the synthesis of UDP-MurNAc-pentapeptide from UDP-*N*-acetylglucosamine (UDP-GlcNAc).[8−13] At least seven enzymes are required for this process. Two enzymes, MurA and MurB convert UDP-GlcNAc to UDP-MurNAc;[8,9] the other enzymes are ligases that form the amide bonds of the peptide chain, including the bond to the C3 lactate moiety.[10−13] Stage II of peptidoglycan biosynthesis takes place on the cytoplasmic surface of the bacterial membrane and involves the action of two enzymes.[14,15] The first of these enzymes, MraY, catalyzes a pyrophosphate exchange reaction in which phospho-MurNAc-

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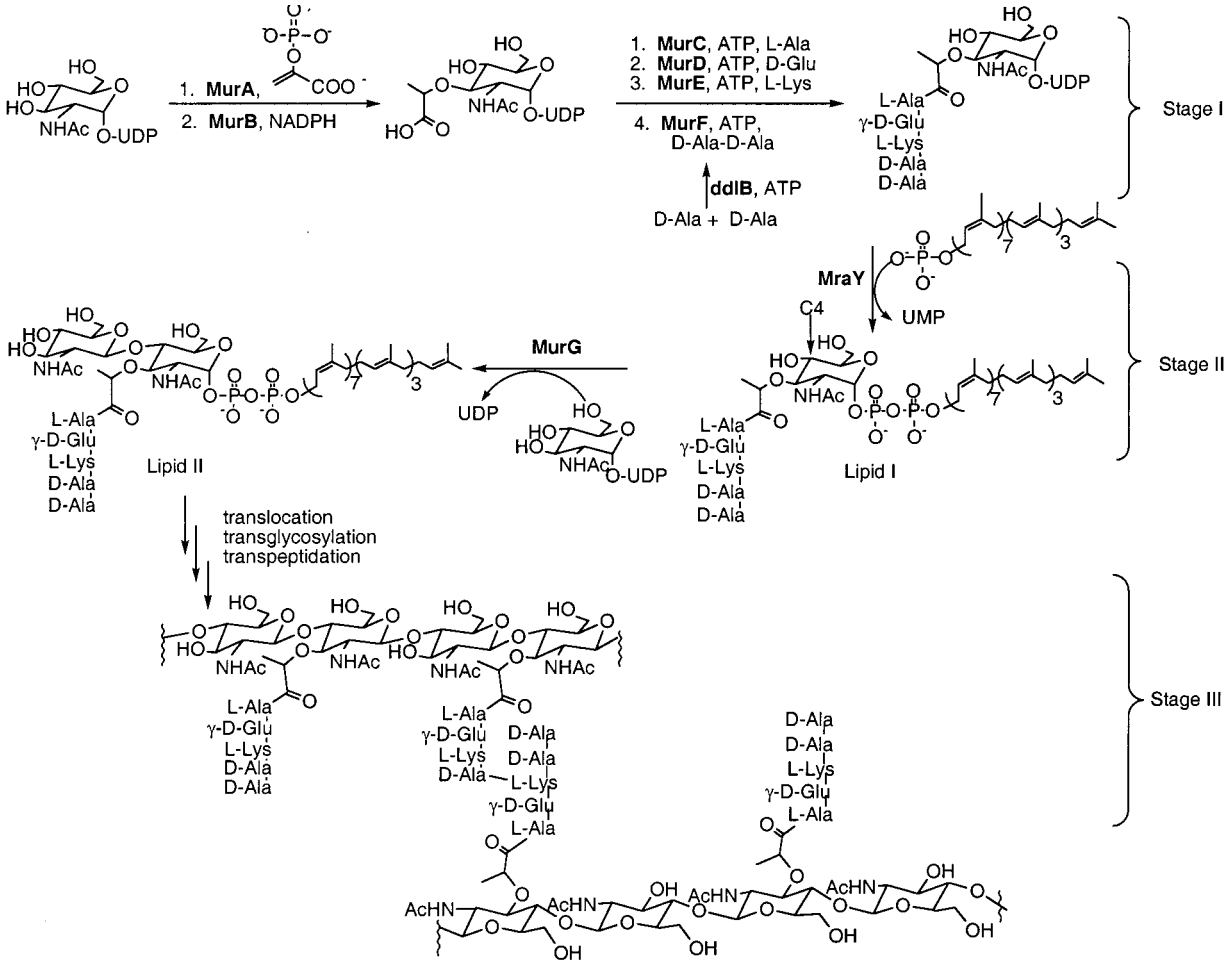
**Scheme 1**




pentapeptide is coupled to a membrane-anchored undecaprenyl ($C_{55}$) phosphate to form an undecaprenyl-pyrophosphoryl-muramyl pentapeptide (Lipid I) with ejection of UMP. The second enzyme, MurG, catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) from UDP to the C4 hydroxyl of the lipid-linked MurNAc pentapeptide. Stage III involves the translocation of this lipid-linked disaccharide to the extracellular face of the cell membrane and its subsequent polymerization and cross-linking by various transglycosylases and transpeptidases.[7]

Rapid progress has been made in understanding the enzymes involved in stage I of peptidoglycan biosynthesis. All of the stage I enzymes are soluble and many of the stage I substrates are relatively easy to obtain. Mechanistic studies have been carried out on a number of stage I enzymes, and crystal structures of four of these enzymes are already available.[7,8−11,13]

The enzymes involved in stages II and III of peptidoglycan biosynthesis have proven to be more difficult to study than the stage I enzymes. Most of the stage II and III enzymes are membrane-associated, which can make purification for mechanistic and structural analysis significantly more complicated.[16]

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In addition, and perhaps more importantly, substrates to monitor the activity of these enzymes cannot be obtained readily due to low natural abundance and/or unwieldy physical properties.[17]

Many of the problems involved in studying stage II and stage III enzymes could be solved by utilizing synthetic alternative substrates with altered physical properties in place of the natural substrates, to evaluate enzymatic activity. In this paper we report details on the synthesis of a soluble Lipid I substrate analogue and its use to characterize the activity of purified MurG, the GlcNAc transferase that assembles the disaccharide *N*-acetylglucosaminyl-*N*-acetylmuramyl pentapeptide subunit of peptidoglycan.[18] With this substrate analogue we have been able to characterize the kinetic behavior and substrate requirements of MurG in the absence of membranes.

## Background

MurG, a membrane-associated enzyme that converts Lipid I to Lipid II in stage II of peptidoglycan biosynthesis, was first described in the 1960s.[19,20] The gene for the enzyme was cloned in 1980.[21] In the early 1990s the DNA and corresponding amino acid sequences were reported independently by two different groups.[15a,b] Homologues from more than a dozen other microorganisms have now been identified by DNA sequence analysis. Unfortunately, there is no homology between MurG and any proteins for which structural information is available, and nothing is known about its three-dimensional structure. Moreover, no mechanistic investigations of the enzyme have yet been reported because until recently there were no direct assays for MurG activity.

The major problem with studying MurG is that the Lipid I substrate is virtually impossible to isolate from bacterial cells.[17,20] It is present in minute quantities and has a detergent-like structure that makes it hard to handle. Because Lipid I cannot be isolated readily, MurG activity is typically monitored using a coupled enzyme assay involving the *in situ* generation of the substrate.[15c,22] In this assay, bacterial membranes containing MraY, MurG, and undecaprenyl phosphate are incubated with UDP-MurNAc-pentapeptide, which can be readily isolated from bacterial cells, and UDP-($^{14}$C)-GlcNAc. MraY transfers phospho-MurNAc-pentapeptide to undecaprenyl phosphate to form Lipid I, the substrate for MurG. MurG then transfers radiolabeled GlcNAc to Lipid I to form Lipid II. After the reaction is terminated, the lipid fraction is separated from other components in the reaction mixture. The lipid-linked radioactivity is then quantified. While this assay could be useful for evaluating potential MurG inhibitors, it is not adequate for detailed mechanistic or structural analyses of the enzyme. These studies require access to an acceptor substrate that can be used to monitor the activity of purified enzyme under well-defined and controlled conditions.

Auger and co-workers recently described a semisynthetic route to a functional Lipid I substrate analogue containing a 35-carbon lipid chain.[23] In this approach, UDP-MurNAc pentapeptide was enzymatically degraded to phospho-MurNAc-pentapeptide and then chemically coupled to an activated lipid phosphate to form the pyrophosphate linkage. We recently reported a *de novo* synthesis of a water soluble Lipid I substrate analogue containing a 10-carbon lipid chain.[18] Using this substrate analogue we have developed a rapid and efficient assay to monitor the activity of MurG. Below we describe the use of this substrate analogue to evaluate the kinetic properties of MurG.

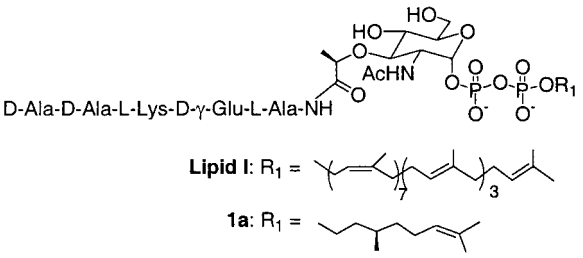


**Figure 1.** Structures of Lipid I and analogue **1a**.

## Results

**Design and Synthesis of the Lipid I Analogue.** We chose to synthesize the substrate analogue *de novo* because having a flexible synthetic route would facilitate access to a range of different compounds in which the individual building blocks of the acceptor substrate are independently varied, permitting us to examine the structural requirements for acceptor recognition. As we did not initially know what would be required for MurG activity, we selected a target identical to the natural Lipid I substrate in everything except the lipid chain (Figure 1). Lipid I contains an undecaprenyl unit α-linked to the anomeric carbon via an allylic pyrophosphate. We were concerned that having a long lipid chain would complicate synthesis, purification, and handling of the substrate; we were also concerned about the potential instability of the allylic pyrophosphate linkage.[24] Therefore, we replaced the undecaprenol with a citronellol group. Although citronellol is only one-fifth as long as undecaprenol and lacks the double bond in the first isoprene unit, we did not expect these changes to destroy enzyme activity because the reaction that MurG catalyzes involves the C4 hydroxyl of the MurNAc-pentapeptide, which is quite far from the lipid anchor. Moreover, biochemical work on the enzyme had suggested that although it contacts the surface of the membrane, it does not actually penetrate into the lipid bilayer where the lipid portion of the substrate is located.[15f]

The synthesis of the substrate analogue is shown in Scheme 2. The synthetic approach is conceptually straightforward and involves the assembly of the three fragments, the sugar, the peptide, and the phospholipid, using standard peptide and phosphodiester bond forming reactions. The C3 lactate of readily available **2** was protected as the trichloroethylester and then hydrogenated to remove the anomeric benzyl group.[25] After reattachment of the 4,6-benzylidene acetal to produce **4**, the anomeric hydroxyl was treated with *i*Pr$_2$NP(OBn)$_2$ to make the phosphite, which was oxidized *in situ* to produce the dibenzyl phosphate as a 4:1 α:$\beta$ anomeric mixture (81% yield).[26] To

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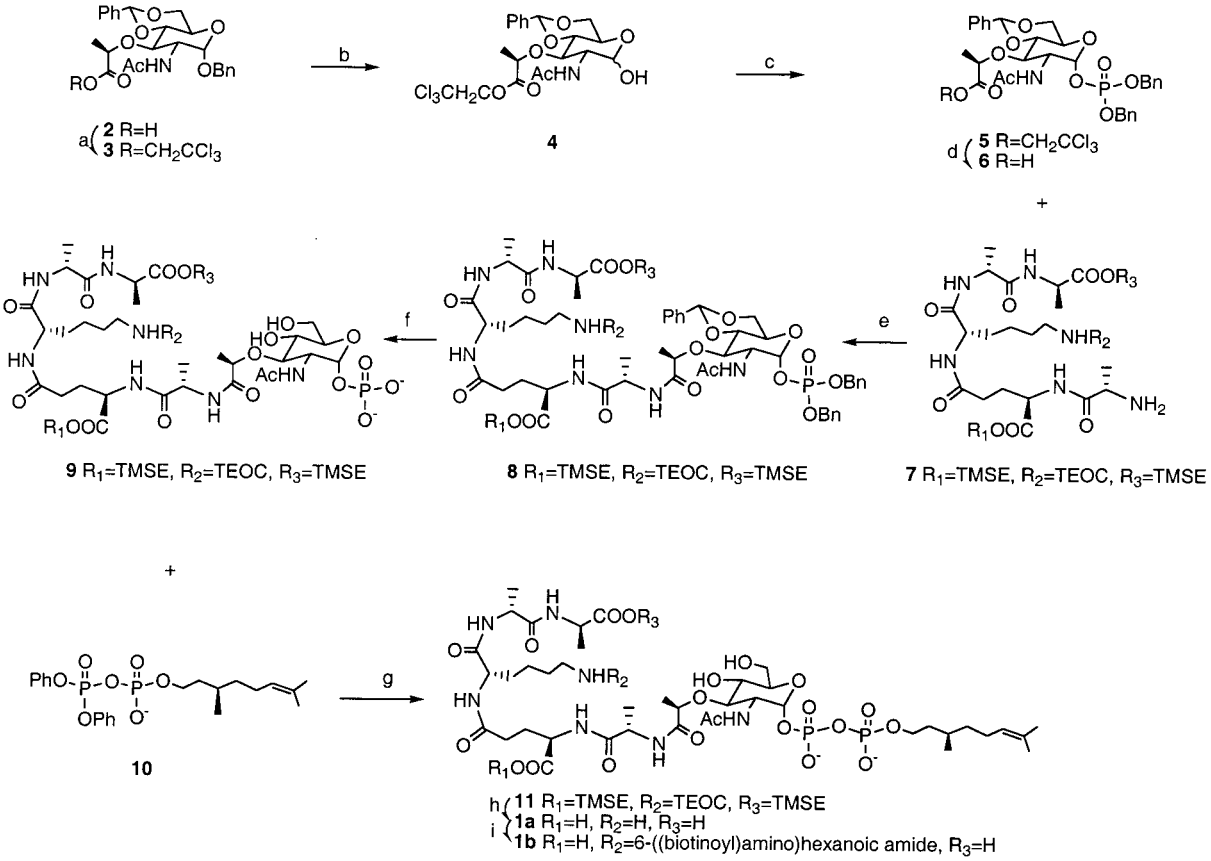
**Scheme 2**[a]




[a] Reagents and conditions: (a) $CCl_3CH_2OH$, DCC/DMAP, THF, rt, 4 h, 80%; (b) 1. $H_2Pd$, EtoAc, rt, 0.5 h; 2. $PhCH(OCH_3)_2$, cat. TsOH, DMF, rt, 10 h, 81%, 2 steps; (c) $iPr_2NP(OBn)_2$, $^1H$-tetrazole, $CH_2Cl_2$, −30 °C → 0 °C, 0.5 h, then mCPBA, −40 °C → 25 °C, 2 h, 70%; (d) Zn dust, 90% AcOH/$H_2O$, rt, 1 h, 91%; (e) HOBt, PyBop, DIEA, DMF, 0 °C, 30 min, 87%; (f) $H_2Pd$, $CH_3OH$, rt, 30 min, then pyridine; (g) $CH_2Cl_2$, py, rt, 18 h, 68%; (h) TBAF, DMF, rt, 24 h, 93%; (i) 6-((biotinoyl)amino)hexanoic acid succinimide ester, $NaHCO_3$, $H_2O$/dioxane, rt, 2 h, 80%.

attach the peptide chain the lactate moiety of the sugar was deprotected by reductive cleavage and then coupled to the protected pentapeptide **7**. The glycopeptide was prepared for coupling to the lipid chain by hydrogenolysis of the dibenzyl phosphate, which simultaneously removed the 4,6-benzylidene to give **9**. Subsequent coupling to lipid derivative **10**, generated *in situ* by treating citronellol phosphate with diphenyl chlorophosphate,[24,27] proceeded smoothly in the presence of the free hydroxyls at C4 and C6 to produce **11**. Finally, the protecting groups on the peptide were removed with fluoride ion.

The methods that have been used to detect product in previous MurG activity assays rely on the differential lipophilicity of the lipid II product and the UDP-GlcNAc starting material, and involve time-consuming separations using, e.g., paper chromatography or C18 chromatography. To facilitate more rapid detection, we attached a biotin label to the lysine side chain of the MurNAc sugar to obtain substrate analogue **1b**, as shown in Scheme 2. The biotin label permits an avidin affinity-based separation of the radiolabeled disaccharide product from radiolabeled UDP-GlcNAc.[28,29] Because it was known that *Escherichia coli* MurG accepts Lipid I substrates containing fluorescent labels on the side chain of the third amino acid, we did not think that the biotin label would impede substrate recognition.[30]

The preceding synthesis is convergent and permits the independent variation of the peptide, the lipid, and the carbohydrate to examine the roles of different structural features in acceptor recognition. For the studies reported below we have synthesized a small set of other acceptor analogues with changes to the lipid and the peptide (Figure 2). It should be noted that Hitchcock *et al.* have recently reported a nice synthesis of UDP-MurNAc pentapeptide, the Park nucleotide, which is the substrate for MraY.[31] Hitchcock's synthetic approach is similar to ours, differing chiefly in the choice of protecting groups and the conditions required for removal. Hence, two different sets of conditions are available for making derivatives of these highly functionalized substrates, permitting a wide range of targets to be made.

**The Biotin-Capture Assay.** Using the cell lysate from a bacterial culture overexpressing MurG as the enzyme source, we previously established using a biotin-capture assay that substrate analogue **1b** is a functional GlcNAc acceptor.[18] Our assay for detecting MurG activity is outlined in Scheme 3. Substrate analogue **1b** and UDP-($^{14}$C)-GlcNAc are incubated with a source of MurG enzyme for a period of time and then quenched by adding SDS to a final concentration of 0.33% (w/v). An avidin-derivatized resin is added to the mixture to

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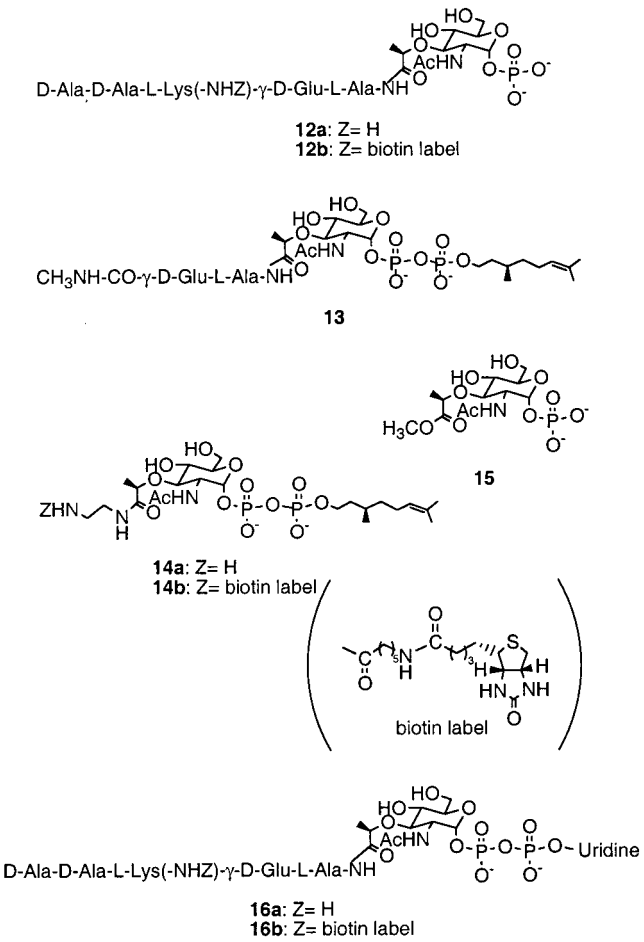


**Figure 2.** Alternative substrates and substrate-based inhibitors of MurG activity.

sequester biotin-labeled material. After washing away unbound radioactivity, the bound radioactivity is counted and the velocity is determined from the counts incorporated as a function of time. In the absence of an active source of MurG or the biotinylated acceptor **1b**, the radioactivity associated with the avidin resin can be washed to background levels (<100 cpm). Hence, the noise in the biotin-capture assay is very low even when crude bacterial lysates are used as an enzyme source.

Due to the expense of the components, the reaction volumes we use are small, typically 20 $\mu$L. This permits us to use submicrogram quantities of biotinylated substrate in the reactions. In order for accurate product quantitation, it is important to have an excess of biotin binding sites to biotin-labeled substrate. We adjust the amount of avidin resin depending on the concentration of **1b** used in the reactions to achieve a 3-fold nominal excess of biotin binding sites to biotin-labeled compound. Because the washing is efficient, differences in the amount of resin added do not significantly affect product quantitation. Therefore, it is possible to span a broad range of substrate concentrations for kinetic analysis.[32]

**MurG Purification**. With a direct activity assay for MurG in hand, we were able to undertake the purification and characterization of the enzyme. MurG was expressed from a pET3a vector in a BL21(DE3)pLysS host.[33,34] SDS-PAGE analysis of the cell lysate showed an intense band at a molecular weight around 38 kDa. Although it has been reported that MurG is membrane-associated,[15f] we found that a significant fraction of MurG partitions into the soluble (cytoplasmic) fraction. The proportion of MurG that was soluble increased upon addition of 3% (v/v) Triton X-100 (Figure 3a). By using the biotin-capture assay described above, we established that the protein in the soluble fraction is active, suggesting that purifying the protein to homogeneity in active form might be easier than anticipated.

MurG has an estimated isoelectric point of 9.6 so we purified it at pH 6.0 on a strong cation exchange (SP-Sepharose) column. Following elution with a NaCl gradient, the protein was concentrated and passed over a Superdex 200 size exclusion column. MurG eluted in a sharp peak with a retention time corresponding to a molecular weight of 76 kDa, indicating that it purifies as a dimer (Figure 3b). The structural determinants of dimer formation are not yet understood, nor is the functional significance. We note, however, that a number of other glycosyltransferases have been reported to be dimers or higher order oligomers.[35] Analysis of the purified MurG dimer on a Coomassie blue-stained, denaturing polyacrylamide gel showed a single band around 38 kDa (Figure 3a). The purity was estimated to be greater than 95%.[36] The yield of purified enzyme was approximately 9 mg/L of bacterial culture.

Preliminary experiments to determine suitable conditions for studying MurG showed that the purified enzyme has good activity in HEPES buffer. A study of the pH dependence of the activity showed that MurG functions in the pH range 6.5−9.5, with optimal activity at pH 8.3. The addition of $Mg^{2+}$, $Mn^{2+}$, or $Ca^{2+}$ at concentrations of 2.5−5 mM increases the activity in HEPES by 30−50%. The addition of EDTA abolishes enzymatic activity. $Ni^{2+}$ and $Zn^{2+}$ were found to be strongly inhibitory (Figure 4). In combination, these results show that MurG, like many other glycosyltransferases that have been characterized,[37−39] requires for optimal activity an oxophilic divalent cation that forms six-coordinate complexes. The studies reported below were carried out in HEPES buffer, pH 7.9, supplemented where noted with 5 mM $MgCl_2$ or 2.5 mM $MnCl_2$.

**Kinetic Parameters.** Kinetic parameters can be determined by simultaneously varying the concentrations of both substrates and constructing double reciprocal plots of velocity versus substrate concentration. Preliminary experiments were carried

(32) It is obvious that the biotin-capture assay described can also be adapted for a scintillation proximity assay. We have found that MurG will transfer radiolabeled GlcNAc to biotinylated substrate **1b** that is already bound to the avidin resin, raising the possibility of developing a continuous scintillation proximity assay.

(33) The *murg* gene was subcloned into the pET3a vector from a pET15b:*murg* plasmid generously supplied by Ms. Sunita Midha of Transcell Technologies. The sequence of this gene was found to be identical to that previously reported (ref 15a,b) except for the presence of an additional ATG codon at the 5′ end, which was introduced during construction of the pET15b:murg plasmid. The N-terminal sequence of the expressed protein is MMSGQG....We chose not to remove the additional methionine because it appears to increase the level of expression of the protein.

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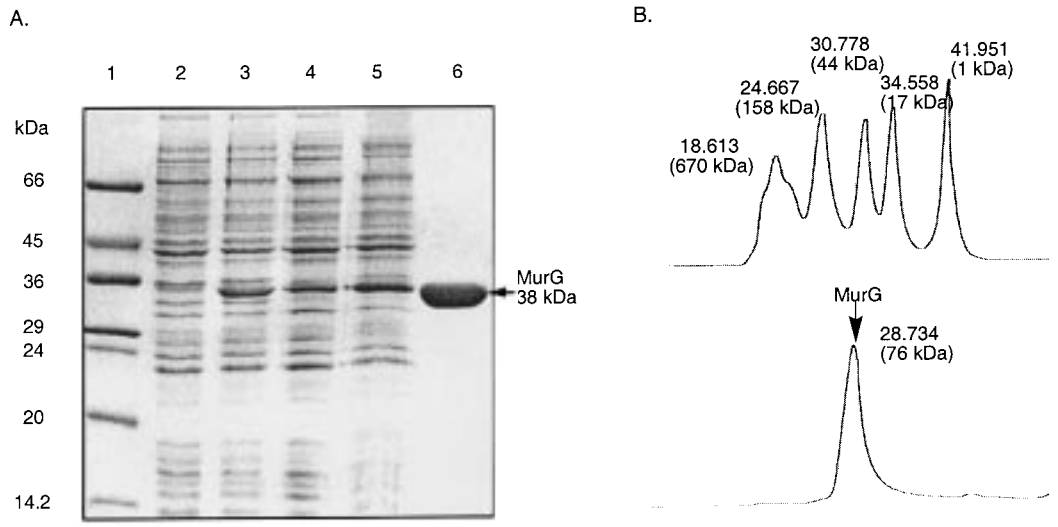
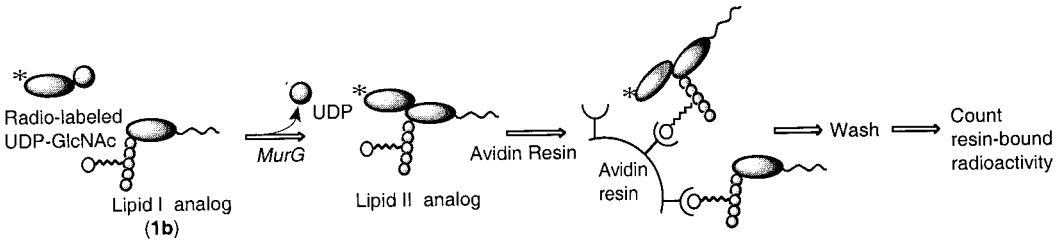



**Figure 3.** (A) SDS−PAGE of MurG (38 kDa). Lane 1: Molecular weight marker. Lane 2: Uninduced cell lysate. Lane 3: Total cell lysate after 3.5 h induction. Lane 4: Supernatant without detergent. Lane 5: Supernatant with 3% (v/v) Triton X-100. Lane 6: Purified MurG. (B) Elution profiles of molecular weight standard and purified MurG (76 kDa) from a Superdex 200 size exclusion column. Numbers indicate the elution time in minutes. Elution times from different injections agree within 0.1 min. The corresponding molecular weights are shown in parentheses.

**Scheme 3.** Schematic of Biotin-Capture Assay for MurG Activity



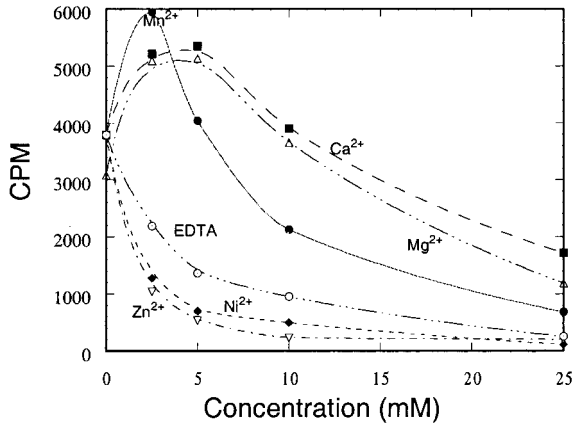




**Figure 4.** Effect of increasing concentrations of divalent metal ions and EDTA on MurG activity. Assays were carried out at 18 $\mu$M **1b** and 34 $\mu$M UDP-GlcNAc.

out to identify appropriate concentration ranges for initial rate experiments. Reactions were run for two to five minutes depending on substrate concentration and the substrate depletion was less than 10% in all cases. Product formation under selected reaction conditions was found to be linear beyond five minutes. Double reciprocal plots of the initial rate data are shown in Figure 5. The lines in the double reciprocal plots converge, indicating that the enzyme utilizes a sequential mechanism in which both substrates bind prior to any bond-forming or breaking process. The slope replot does not pass through the origin, which implies that the enzyme utilizes either a steady-state ordered mechanism or a rapid equilibrium random mechanism.[40]

Kinetic parameters for MurG in the absence of additional metal ions were found to be $K_{\text{UDP-GlcNAc}} = 45 \pm 12$ $\mu$M, $K_{\mathbf{1b}} = 44 \pm 8$ $\mu$M, $V_{\max} = 0.20 \pm 0.04$ $\mu$mole/min/mg, and $k_{\text{cat}} = 7.4 \pm 1.3$ min$^{-1}$. The parameters were $K_{\text{UDP-GlcNAc}} = 58 \pm 30$ $\mu$M, $K_{\mathbf{1b}} = 37 \pm 4$ $\mu$M, $k_{\text{cat}} = 16 \pm 2$ min$^{-1}$, and $K_{\text{UDP-GlcNAc}} = 46 \pm 12$ $\mu$M, $K_{\mathbf{1b}} = 36 \pm 9$ $\mu$M, $k_{\text{cat}} = 19 \pm 4$ min$^{-1}$ in the presence of 5 mM $MgCl_2$ and 2.5 mM $MnCl_2$, respectively.[41] Hence, as with other metal-ion dependent glycosyltransferases, the added metal ion seems to affect catalysis rather than substrate binding.[42] The $k_{\text{cat}}$ values of MurG are within the range reported for other glycosyltransferases, which typically have low turnover numbers: for example, 10−40 min$^{-1}$ for α-1,3-fucosyltransferase V; 10 min$^{-1}$ for human $\beta$-1,4-galactosyltransferase, 0.2

(40) We have not yet been able to determine the precise mechanism. UDP is a noncompetitive inhibitor of the acceptor substrate and a competitive inhibitor of the UDP-GlcNAc donor, implying that both UDP and UDP-GlcNAc bind to the free form of the enzyme at the same site. This rules out a compulsory-ordered mechanism in which the acceptor substrate binds first. None of the acceptor analogues reported here are both competitive inhibitors of **1b** and incapable of reacting, and we cannot complete the mechanistic analysis until we obtain suitable inhibitors. See: (a) Fromm, H. J. *Methods Enzymol.* **1979**, *63*, 467−486. Both types of mechanisms have been observed in other glycosyltransferases. See: (b) Qiao, L.; Murray, B. W.; Shimazaki, M.; Schultz, J.; Wong, C.-H. *J. Am. Chem. Soc.* **1996**, *118*, 7653−7662. (c) Palcic, M. M.; Heerze, L. D.; Srivastava, O. P.; Hindsgaul, O. *J. Biol. Chem.* **1989**, *264*, 17174−17181. Additional compounds are being made to probe the mechanism further.

(41) The observed $k_{\text{cat}}$ values, which are based on a molecular weight of 38 kDa for the active enzyme and an assumption of full activity based on the nominal concentration, could be underestimated. The enzyme is purified as a dimer with a molecular weight of ∼76 kDa; furthermore, control experiments have shown that it loses activity rapidly upon exposure to new surfaces—as upon dilution and transfer.

(42) Beyer, T. A.; Hill, R. L. *J. Biol. Chem.* **1980**, *255*, 5373−5379.

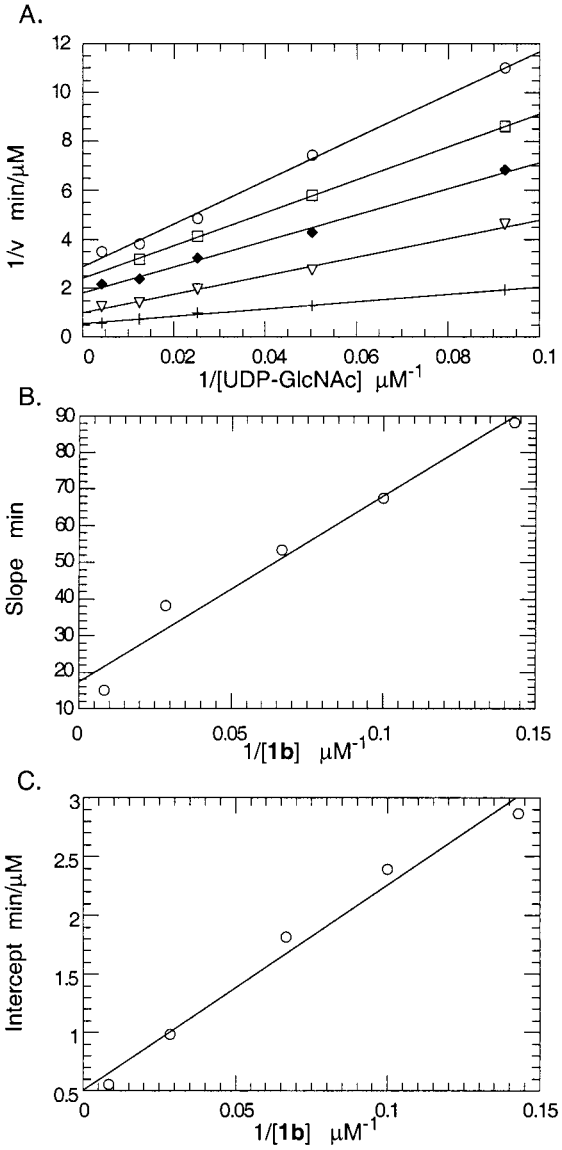



**Figure 5.** (A) Double reciprocal plots of the initial rate data with respect to UDP-GlcNAc as the varied substrate. Initial rates were measured at fixed acceptor **1b** concentrations of 7 $\mu$M (○), 10 $\mu$M (□), 15 $\mu$M (◆), 35 $\mu$M (▽) and 120 $\mu$M (+). (B) Secondary plots of slope and (C) intercept vs 1/[**1b**]. Data shown were obtained in the absence of metal ion. Analysis of the data yields the following kinetic parameters: $K_{\text{UDP-GlcNAc}} = 45 \pm 12$ $\mu$M, $K_{\mathbf{1b}} = 44 \pm 8$ $\mu$M, $k_{\text{cat}} = 7.4 \pm 1.3$ min$^{-1}$. Similar studies were carried out in the presence of 5 mM $MgCl_2$, yielding $K_{\text{UDP-GlcNAc}} = 58 \pm 30$ $\mu$M, $K_{\mathbf{1b}} = 37 \pm 4$ $\mu$M, $k_{\text{cat}} = 16 \pm 2$ min$^{-1}$; and 2.5 mM $MnCl_2$ yielding: $K_{\text{UDP-GlcNAc}} = 46 \pm 12$ $\mu$M, $K_{\mathbf{1b}} = 36 \pm 9$ $\mu$M, $k_{\text{cat}} = 19 \pm 4$ min$^{-1}$.

min$^{-1}$ for xylosyltransferase, and 1200 min$^{-1}$ for GlcNAc transferase II.[37,43,44]

**Acceptor Specificity.** In the assay described above, both the donor sugar and the acceptor sugar are labeled, one with a radiolabel and the other with a biotin label. To be detected, products must be both biotinylated and radiolabeled. Therefore, it is possible to evaluate rapidly the structural determinants of substrate recognition by comparing the concentrations at which a series of unlabeled substrate analogues inhibit the incorporation of radioactivity into the avidin-coated beads by 50% ($IC_{50}$ values). The $IC_{50}$ values of a variety of synthetic acceptor analogues were evaluated and the results are shown in Table 1.

**Table 1.** $IC_{50}$ Values and Relative Rates for Selected Compounds

| acceptor analogues | $IC_{50}$[a] | relative rate, %[b] |
|---|---|---|
| **1a** | 40 ± 3 $\mu$M | |
| **1b** | | 100 |
| **12a** | 60 ± 30 $\mu$M | |
| **12b** | | *c* |
| **13** | 280 ± 50 $\mu$M | |
| **14a** | 470 ± 70 $\mu$M | |
| **14b** | | 0.3 |
| **15** | 1.4 ± 0.1 mM | |
| **16a** | 13 ± 3 $\mu$M | |
| **16b** | | 0.2 |

| donor analogues | $IC_{50}$[a] |
|---|---|
| UDP | 65 ± 1 $\mu$M |
| UMP | 600 ± 50 $\mu$M |
| Uridine | >1 mM |
| GDP | 870 ± 100 $\mu$M |
| CDP | 560 ± 180 $\mu$M |
| ADP | 1.3 ± 0.3 mM |
| GlcNAc-1′-phosphate | >1 mM |
| UDP-Glc | >1 mM |
| UDP-GaINAc | >1 mM |
| UDP-glucuronic acid | 470 ± 120 $\mu$M |

[a] All $IC_{50}$ assays were performed under the same conditions, with 18 $\mu$M 1b and 34.3 $\mu$M UDP-GlcNAc. Each $IC_{50}$ value was determined by fitting five or six data points to equation: $v_i/v_o = 1/(1 + [I]/IC_{50})$ where $v_i$ is the initial rate in the presence of inhibitor at concentration [I], and $v_0$ is the initial rate without inhibitor. [b] Relative rates for alternative acceptors were obtained using: 40 $\mu$M UDP-MurNAc, 40 $\mu$M acceptor, and 0.4 $\mu$g MurG in a total of 20 $\mu$L reaction mixture. [c] Under the above conditions, no product was detected; at extremely high enzyme concentrations (10 $\mu$g), some product can be detected after a half hour.

The nonbiotinylated substrate analogue **1a** was found to have an $IC_{50} = 40 \pm 3$ $\mu$M under the assay conditions. This compound, which is assumed to have comparable activity to the corresponding biotinylated substrate, provides a baseline for comparing other analogues. As shown in Table 1, the $IC_{50}$ values increase by an order of magnitude as the peptide chain is truncated from five residues to a simple diamine derivative (compare **1a**, **13**, **14a**), indicating that the enzyme is sensitive to the length of the peptide chain. By contrast, removing the citronellol-phosphate tail or replacing it with uridine monophosphate does not affect inhibitory potency (compare **1a**, **12a**, and **16a**). The $IC_{50}$ of phospho-MurNAc-pentapeptide compound **12a** is comparable to that of the nonbiotinylated substrate analogue **1a**. Removing the peptide and the citronellol-phosphate tail (see **15**) abolishes inhibitory potency.

The $IC_{50}$ assay provides rapid information on inhibitory potency but does not distinguish between compounds that function as alternative substrates, competing with the biotinylated acceptor for the radiolabeled GlcNAc, and those that function as dead-end inhibitors. Selected compounds were biotinylated to evaluate their ability to function as acceptors with purified MurG. The biotinylated diamine derivative analogue **14b** did function as an acceptor, albeit a much less efficient one than the **1b**, consistent with its poor ability to inhibit the enzymatic reaction. The monophosphate analogue **12b**, in contrast, is a good inhibitor but has almost undetectable acceptor activity. To try to distinguish the relative importance of the second phosphate group and the lipid chain in acceptor activity, we tested biotinylated UDP-MurNAc-pentapeptide **16b** as an alternative substrate. This compound was a modest acceptor, comparable to **14b.** The better acceptor activity of **16b** compared to **12b** suggests that the pyrophosphate plays a

(43) Bendiak, B.; Schachter, H. *J. Biol. Chem.* **1987**, *262*, 5784−5790.
(44) Kearns, A. E.; Campbell, S. C.; Westley, J.; Schwartz, N. B. *Biochem.* **1991**, *30*, 7477−7483.

role in acceptor recognition, perhaps by helping to orient the acceptor in the binding pocket. However, the enzyme is also sensitive to the group that is attached to the pyrophosphate. It is not yet clear whether a lipid chain itself is necessary; this question is being examined further.[45] Although biotinylated UDP-MurNAc-pentapeptide **16b** is a poor acceptor compared with **1b**, it is considerably easier to obtain by isolation from natural sources and could prove useful for monitoring the activity of purified MurG.

**Donor Specificity.** Several commercially available donor analogues were investigated to probe the requirements for donor recognition. As shown in Table 1, the enzyme is sensitive to both the identity of the base and the diphosphate linkage. UDP is a significantly better inhibitor than either UMP or any other nucleotide diphosphates. The enzyme also shows high specificity for the equatorial stereochemistry at the C4 position of the donor. Thus,UDP-GalNAc, which differs from UDP-GlcNAc only in having an axial hydroxyl at C4, did not inhibit the enzyme significantly even at mM concentrations. Furthermore, it showed minimal donor activity. It is possible that MurG has evolved to select stringently for UDP-GlcNAc over UDP-GalNAc since the equatorial C4 hydroxyl of GlcNAc is the nucleophile in the subsequent enzyme-catalyzed polymerization of the disaccharide.

UDP-glucose was also a poor inhibitor, suggesting that the C2 *N*-acetyl group plays a key role in donor recognition. However, UDP-glucuronic acid, which contains a C2 hydroxyl and a C6 carboxylate, was found to have an $IC_{50}$ of $\sim$500 $\mu$M, making it one of the better donor-based inhibitors. An analysis of the primary sequence of MurG has revealed homology between a region in the C-terminal half of MurG, spanning residues 240−315, and a region in the C-terminal half of the UDP-glucuronosyltransferases.[46] The UDP-glucuronic acid binding site is believed to be located in the C-terminal half of the glucuronosyltransferases.[47] Structural similarities between the donor binding site of MurG and that of the glucuronosyltransferases may explain the ability of UDP-glucuronic acid to inhibit MurG activity. If so, this would imply that the C-terminal half of MurG is involved in the donor binding.

## Discussion and Conclusion

The studies reported above establish the utility of water soluble Lipid I substrate analogues to study the kinetic properties and specificity of MurG. In its native context MurG operates at a membrane surface and utilizes a membrane-bound Lipid I acceptor; however, for many mechanistic investigations it may be preferable to leave out membranes. Membranes impose constraints on substrate recognition that can complicate the analysis of results. For example, compounds that do not partition into membranes are unlikely to compete effectively with a membrane-bound substrate for a membrane-associated enzyme even if they are highly complementary to the binding pocket. Therefore, it would not be possible using a membrane assay alone to distinguish between compounds that do not bind the enzyme well because they do not make the appropriate set of active-site contacts and compounds that do not anchor appropriately in membranes. The solution assay provides direct information on active-site topology. An additional advantage of working in solution is that the assay is technically easier to carry out. We expect that this will facilitate investigations on mutant proteins constructed to test the roles of various amino acids in activity. Once the properties of the enzyme in solution are understood, the role of the membrane can be addressed. In additon to playing an important role in substrate specificity (e.g., by requiring membrane-bound substrate), the membrane could have a significant effect on enzyme kinetics.[48]

Our preliminary studies on MurG indicate high selectivity for both the UDP-GlcNAc donor and the MurNAc acceptor. In addition to preferring the UDP group strongly over other nucleotides, the enzyme is quite sensitive to the C2 *N*-acetyl group and the stereochemistry at the C4 position of the donor. The stringent selection of the equatorial hydroxyl group at the C4 position on the donor suggests that MurG plays a key role in ensuring the integrity of peptidoglycan biosynthesis. The equatorial C4 hydroxyl is the acceptor for the next enzymes in the pathway, the transglycosylases that couple the *N*-acetyl glucosaminyl-*N*-acetyl muramic acid disaccharide units. UDP-*N*-acetyl galactosamine is also a common sugar donor in bacterial cells. If MurG did not stringently select against this donor, defective peptidoglycan layers could form, compromising the viability of bacterial cells.

MurG is also sensitive to the substituent on the C3 position of the acceptor. The C3 substituent defines *N*-acetyl muramic acid, differentiating it from *N*-acetyl glucosamine. MurNAc is unique to bacteria, and the sensitivity of MurG to this position suggests that it might be possible to develop inhibitors of MurG that exploit this sensitivity and thus do not interfere with eukaryotic *N*-acetylglucosaminyltransferases.

We have also shown that UDP-MurNAc-pentapeptide, the substrate for MraY, functions as an acceptor with soluble MurG, albeit a much less efficient acceptor than the lipid-linked analogue **1b**.[49] UDP-MurNAc-pentapeptide has been estimated to be $\sim$100 times more abundant than Lipid I in bacterial cells.[17] If there were not an additional level of control over substrate recognition in bacterial cells, UDP-MurNAc-pentapeptide might well be able to compete with Lipid I. We think that the membrane itself may provide this additional level of control. MurG is membrane-associated and the Lipid I substrate is membrane bound. UDP-MurNAc-pentapeptide presumably does not partition significantly into membranes, which prevents it from competing with the lipid-linked substrate.

Glycosyltransferases that catalyze the same type of group transfer reaction as MurG—the transfer of an activated sugar nucleotide to a sugar acceptor with inversion of configuration at the anomeric center of the donor—are quite common in eukaryotes, where they play key roles in the assembly and processing of oligosaccharides in the Leloir pathway.[50,51] Although hundreds of different Leloir pathway glycosyltrans-

(45) UDP-MurNAc-pentapeptide is being evaluated as an alternative acceptor.

(46) Sequence similarities between MurG homologues and monogalactosyldiacylglycerol synthases have been noted previously. See: Shimojima, M.; Ohta, H.; Iwamatsu, A.; Masuda, T.; Shioi, Y.; Takamiya, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 333−337.

(47) Pillot, T.; Ouzzine, M.; Fournel-Gigleux, S.; Lafaurie, C.; Tebbi, D.; Treat, S.; Radominska, A.; Lester, R.; Siest, G.; Magdalou, J. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 785−791.

(48) Interfacial catalysis has been extensively studied with phospholipases, which are very sensitive to the presence of membranes. See, for example: Jain, M. K.; Gelb, M. H.; Rogers, J.; Berg, O. G. *Methods Enzymol.* **1995**, *249*, 567−614.

(49) The synthetic lipid analogue **1a** does not aggregate in water even at millimolar concentrations. The NMR resonance lines of this compound in $D_2O$ are sharp and show no signs of the broadening that would indicate aggregation. Therefore, the better activity of the lipid-linked substrates is not believed to be due to the presence of micellular structures in the enzymatic reactions.

(50) Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5365−5422.

(51) Field, M. C.; Wainwright, L. J. *Glycobiology* **1995**, *5*, 463−472.

ferases have been identified,[52,53] there is not yet a single-crystal structure available for any of them.[54] This state of affairs is largely due to problems in obtaining sufficient quantities of soluble, active enzyme for structural analysis. Eukaryotic glycosyltransferases are anchored to membranes by a hydrophobic peptide sequence and they must be engineered for solubility in aqueous buffer.[55] The engineered proteins must then be overexpressed in active form. In general, overexpression of active eukaryotic glycosyltransferases has proven to be difficult. MurG, however, can be readily expressed at high levels in *E. coli*. Because MurG catalyzes the same type of reaction as the Leloir pathway glycosyltransferases, it is not unreasonable to expect similarities with respect to active-site structure and catalysis. MurG may, therefore, be a good model system for understanding other glycosyltransferases. Although MurG functions as a membrane-associated enzyme in its native context, the wild-type protein is soluble and active at high concentrations in aqueous buffer, which will simplify structural and mechanistic studies considerably. The finding that UDP-MurNAc-pentapeptide is an alternative substrate may prove useful for many of these studies.

We note in closing that many of the problems involved in studying enzymes involved in peptidoglycan biosynthesis relate to difficulties in handling insoluble substrates. The synthesis of soluble substrate analogues may permit the development of better assays for studying these other enzymes as well.

## Experimental Section

**General.** All plasmid vectors and competent cells were purchased from Novagen. Tetralink tetrameric avidin resin was purchased from Promega Corp. UDP-($^{14}$C)-GlcNAc and UDP-($^{14}$C)-glucose were purchased from NEN DuPont. UDP-($^{14}$C)-glucuronic acid, GDP, and ADP were purchased from ICN Pharmaceuticals. All amino acids were purchased from BAChem. Unless otherwise stated, all chemicals were purchased from Aldrich or Sigma and used without further purification. Dichloromethane, toluene, benzene, pyridine, diisopropylethylamine, and triethylamine were distilled from calcium hydride under dry argon. Diethyl ether and tetrahydrofuran were distilled from potassium benzophenone under dry argon. DMF, ethyl acetate, and methanol were dried over activated molecular sieves.

Analytical thin-layer chromatography (TLC) was performed on silica gel 60 $F_{254}$ plates (0.25 mm thickness) precoated with a fluorescent indicator. The developed plates were examined under short wave UV light and stained with anisaldehyde or Verghn stain. Flash chromatography was performed using silica gel 60 (230−400 mesh) from EM Science.

NMR spectra were recorded on a JEOL GSX-270 NMR spectrometer or a Varian Inova 500/VNMR spectrometer. Chemical shifts ($\delta$) are reported in parts per million (ppm) downfield from tetramethylsilane. Coupling constants ($J$) are reported in hertz (Hz). Multiplicities are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), apparent triplet (apt), broad singlet (bs), pentet (p), and octet (o).

High-resolution mass spectra (FAB) were obtained by Dr. Ron New at the University of California at Riverside Department of Chemistry Mass Spectrometry Facility. Low resolution mass spectra (ESI) were obtained by Dr. Dorothy Little at Princeton University Department of Chemistry.

**Purification of *E. coli* MurG**. BL21(DE3)pLysS cells (Novagen) overexpressing wild type *E. coli* MurG from a pET3a vector (Novagen) were grown in 11L 2XYT medium supplemented with 100 $\mu$g/mL ampicillin and 34 $\mu$g/mL chloramphenicol. When the $OD_{600\ nm}$ reached 0.6, IPTG was added to a final concentration of 1 mM. The induced cell culture was grown for another 3.5 h, then the cells were spun down in 500 mL batches at 5000 rpm (Beckman RC5B centrifuge) for 10 min and the supernatant was decanted. The cell pellets were frozen at −70 °C. After being thawed at 4 °C, each cell pellet was resuspended in 5 mL resuspension buffer containing 25 mM MES (pH 6.0), 5 mM $MgCl_2$, 4 mM DTT, 3% (v/v) Triton X-100 and 20 $\mu$g/mL DNase, and the suspensions were combined for a total of 110 mL. After shaking for 1 h at 4 °C, the debris was spun down at 39 000 rpm for 30 min. The supernatant was decanted, diluted 6-fold with buffer A (25 mM MES pH 6.0, 4 mM DTT), and applied to a SP-Sepharose column (Pharmacia Biotech) equilibrated with Buffer A. After washing for 50 min with 40% Buffer B (20 mM Tris pH 7.9, 1 M NaCl, 4 mM DTT)/60% Buffer A, the bound enzyme was eluted using a linear salt gradient starting with 40% buffer B/60% buffer A and ending with 100% buffer B over 60 min at a flow rate of 2 mL/min. The eluted enzyme was concentrated then applied to a Superdex 200 HR 16/60 column (Pharmacia Biotech) at a flow rate of 1 mL/min of TBSE buffer (150 mM NaCl, 20 mM Tris pH 7.9, 50 mM EDTA and 4 mM DTT). The protein eluted as a symmetric peak at an estimated molecular weight of 76 kDa. The purity of the enzyme was estimated to be greater than 95% from a Coomassie blue-stained SDS-polyacrylamide gel. The purified enzyme was stored at 4 °C, and was stable for at least one month.

**Initial Rate Measurements.** Enzyme stock was prepared by diluting the purified enzyme with buffer containing 100 mM NaCl, 20 mM Tris PH 8.0, 10 mM EDTA to a final concentration of 0.1 $\mu$g/$\mu$L in a 0.5 mL tube and storing at 4 °C for 2 days prior to performing the assays. Following an initial drop in activity due to its adsorption to the tube walls, the enzymatic activity stabilized. Twenty-five reactions were prepared by individually mixing 2 $\mu$L of 10X reaction buffer (500 mM HEPES and 50 mM $MgCl_2$) with an appropriate amount of biotinylated aqueous Lipid I analogue (**1b**), radioactive UDP-GlcNAc, nonradioactive UDP-GlcNAc, and sterile deionized $H_2O$ to a final volume of 18 $\mu$L. The final concentrations for the Lipid I analogue (**1b**) were 7 $\mu$M, 10 $\mu$M, 15 $\mu$M, 35 $\mu$M, 120 $\mu$M, and for UDP-GlcNAc 10.8 $\mu$M, 19.9 $\mu$M, 39.8 $\mu$M, 80.2 $\mu$M, 240.5 $\mu$M. Reactions were initiated by adding 2 $\mu$L of the enzyme stock and were run for 4 min at 24 °C. Reactions were stopped by adding 10 $\mu$L of 1% (w/v) SDS.

Radiolabeled product was separated from radiolabeled starting material by incubating each reaction with a 3-fold molar excess of biotin-binding TetraLink Tetrameric Avidin Resin (Promega). Deionized $H_2O$ was added to each tube to a final volume of approximately 250 $\mu$L, and the suspension was transferred to a 1.2 $\mu$m pore size 96-well filter plate fitted to a vacuum-line fitted MultiScreen Assay System (Millipore). The resin was washed 15 times with 0.2 mL of deionized $H_2O$. Washed resin was transferred to a scintillation vials containing 10 mL of Ecolite scintillation fluid each and shaken. Samples were counted immediately on a Beckman LS5000 scintillation counter.

**$IC_{50}$ Measurements**. The $IC_{50}$ assays were performed the same way as the initial rate assays except that the Lipid I analogue (**1b**) and UDP-GlcNAc concentrations were fixed at 18 $\mu$M and 34.3 $\mu$M, respectively. Each set of assays were carried out at five or six different concentrations of one of the inhibitory compounds. The $IC_{50}$ was determined by fitting the data to the following equation:

$$v_i/v_o = 1/(1 + [I]/IC_{50})$$

(52) (a) Wong, C.-H.; Ichikawa, Y.; Krach, T.; Narvor, C. G.; Dumas, D. P.; Look, G. C. *J. Am. Chem. Soc.* **1991**, *113*, 8137−8145. (b) Krezdorn, C. H.; Watzele, G.; Kleene, R. B.; Ivanov, S. X.; Berger, E. G. *Eur. J. Biochem.* **1993**, *212*, 113−120. (c) Ram, B. P.; Munjal, D. D. *CRC Crit. Rev. Biochem.* **1985**, *17*, 257−311. (d) Palcic, M. M.; Ripka, J.; Kaur, K. J.; Shoreibah, M.; Hindsgaul, O.; Pierce, M. *J. Biol. Chem.* **1990**, *265*, 6759−6769. (e) Giordanengo, V.; Bannwarth, S.; Laffont, C.; Van Miegem, V.; Harduin-Lepers, A.; Delannoy, P.; Lefebvre, J. C. *Eur. J. Biochem.* **1997**, *247*, 558−566. (f) Kono, M.; Ohyama, Y.; Lee, Y. C.; Hamamoto, T.; Kojima, N.; Tsuji, S. *Glycobiology* **1997**, *7*, 469−479.

(53) Transferases that have been studied include galactosyltransferases,[52a−c] fucosyltransferases[38,42] *N*-acetylglucosaminyltransferases,[52d] and sialyltransferases.[52e,f] Mechanistic studies have been reported for some glycosyltransferases. See, e.g.: (a) Murray, B. W.; Wittmann, V.; Burkart, M. D.; Hung, S.-C.; Wong, C.-H. *Biochemistry*, **1997**, *36*, 823−831. (b) Kim, S. C.; Singh, A. N.; Raushel, F. M. *Arch. Biochem. Biophys.* **1988**, *267*, 54−59. (c) Bruner, M.; Horenstein B. A. *Biochemistry*, **1998**, *37*, 289−297.

(54) A crystal structure of a monomeric prokaryotic glycosyltransferase (SpsA) was recently reported. See: Charnock, S. J.; Davies, G. J. *Biochemistry* **1999**, *38*, 6380−6385.

(55) (a) Colley, K. J. *Glycobiology* **1997**, *7*, 1−13. (b) Paulson, J. C.; Colley, K. J. *J. Biol. Chem.* **1989**, *264*, 17615−17618.

**Metal Ion Dependence.** Metal ion dependence studies were carried out the same way as the initial rate assays except for the following: (1) The Lipid I analogue (**1b**) and UDP-GlcNAc concentrations were fixed at 18 $\mu$M and 34.3 $\mu$M, respectively. (2) Prior to the assays, 50 mM stock solutions of $ZnCl_2$, $CaCl_2$, $NiCl_2$, $MgCl_2$, and $MnCl_2$ in deionized water and 10X reaction buffer containing only 500 mM HEPES (pH 7.9) in deionized water were prepared. For each metal ion, five reactions were performed at the final metal concentrations of 0, 2.5, 5.0, 10, and 25 mM. A set of five reactions with the final EDTA concentrations of 0, 2.5, 5.0, 10, and 25 mM were carried out the same way as the metal-ion titrations. Reactions were initiated by adding enzyme stock solution to the reaction mixtures, and stopped and analyzed as described above.

**pH Dependence.** The activity of the enzyme (stock concentration 0.06 mg/mL) was tested over a pH range of 6.5 to 9.6. HEPES/$Mg^{2+}$ buffer (50 mM HEPES, 5 mM $MgCl_2$) was used for reactions at pH 6.5, 7.0, 7.5, 8.0, 8.3, 8.5, and 8.7. CHES (2-[*N*-cyclohexylamino]-ethanesulfonic acid)/$Mg^{2+}$ buffer (80 mM CHES, 5 mM $MgCl_2$) was used for pH 8.3, 8.7, 9.0, 9.2, 9.6. All reactions were performed in triplicate, with the Lipid I analogue (**1b**) and UDP-GlcNAc concentrations fixed at 18 $\mu$M and 34.3 $\mu$M, respectively. Reactions were run for 3 min in HEPES buffers and 4 min in CHES buffers to yield comparable percent conversion at pH 8.3 and 8.7.

**Synthesis of Substrate Analogues. Compound 3.** To a solution of compound **2** (482 mg, 1.02 mmol) and 4-(dimethylamino)pyridine (10 mg, 0.08 mmol) in 8 mL of THF was added trichloroethanol (0.23 mL, 2.40 mmol) followed by 1,3-dicyclohexylcarbodiimide (248 mg, 1.20 mmol). After stirring at room temperature for 4 h, the reaction solution was filtered through a cotton plug, and the precipitate was rinsed with EtOAc. The filtrate was concentrated and purified by flash chromatography (15% EtOAc/$CH_2Cl_2$) to give 453 mg (80%) of **3** as a white powder: $R_f$ 0.39 (15% EtOAc/$CH_2Cl_2$); $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 7.43−7.25 (m, 10 H), 7.07 (d, $J$ = 6.0 Hz, 1 H), 5.59 (s, 1 H), 5.34 (d, $J$ = 3.2 Hz, 1 H), 4.98 (d, $J$ = 11.9 Hz, 1 H), 4.68(d, $J$ = 12.0 Hz, 1 H), 4.66 (q, $J$ = 7.0 Hz, 1 H), 4.60 (d, $J$ = 11.9 Hz, 1 H), 4.51 (d, $J$ = 12.0 Hz, 1 H), 4.21 (dd, $J$ = 10.5, 4.8 Hz, 1 H), 4.00 (m, 1 H), 3.85 (m, 2 H), 3.75 (m, 2 H), 2.04 (s, 3 H), 1.50 (d, $J$ = 7.0 Hz, 3 H); $^{13}$C NMR ($CDCl_3$, 500 MHz) $\delta$ 173.8, 170.9, 137.5, 137.4, 129.3, 128.6, 128.5, 128.1, 128.0, 126.1, 101.6, 97.5, 94.6, 83.4, 75.2, 75.1, 74.3, 70.5, 69.2, 63.1, 54.2, 23.4, 18.9; HRMS(FAB) calcd for $C_{27}H_{31}NO_8Cl_3$ [M + $H^+$]: 602.1115, found: 602.1130.

**Compound 4.** To a solution of compound **3** (360 mg, 0.60 mmol) in 30 mL of EtOAc was added 500 mg of 20% Pd−C. The reaction vessel was filled with hydrogen. After stirring at room temperature for 30 min, the suspension was filtered, and the catalyst was rinsed with methanol. The filtrate was concentrated to give a clear oil which was used in the next reaction without further purification.

To a solution of this clear oil in 6 mL of DMF was added benzylaldehyde dimethyl acetal (0.9 mL, 6.0 mmol) followed by *p*-toluenesulfonic acid (11.4 mg, 0.06 mmol). The reaction was stirred at room temperature for 10 h and neutralized with saturated $NaHCO_3$. Then the mixture was extracted with $CH_2Cl_2$ (3 × 20 mL). The $CH_2Cl_2$ layers were combined, dried over anhydrous sodium sulfate, filtered, concentrated, and purified by flash chromatography (90% EtOAc/petroleum ether) to give 248 mg (81%) of **4** as a mixture of α, $\beta$ anomers (α:$\beta$ = 4:1): $R_f$ (α anomer) 0.33, $R_f$ ($\beta$ anomer) 0.28 (90% EtOAc/petroleum ether); α anomer $^1$H NMR ($CDCl_3$, 270 MHz) $\delta$ 7.50−7.35 (m, 5 H), 5.66 (bs, 1 H), 5.58 (s, 1 H), 5.02 (d, $J$ = 12.0 Hz, 1 H), 4.95 (m, 1 H), 4.67 (m, 1 H), 4.58 (d, $J$ = 12.0 Hz, 1 H), 4.27 (dd, $J$ = 10.0, 5.0 Hz, 1 H), 4.05 (m, 1 H), 2.06 (s, 3 H), 1.52 (d, $J$ = 7.0 Hz, 3 H); $^{13}$C NMR ($CDCl_3$, 270 MHz) $\delta$ 174.2, 171.9, 137.6, 129.2, 128.5, 126.2, 101.5, 94.7, 91.4, 83.5, 75.5, 75.0, 74.6, 69.2, 62.9, 54.9, 23.4, 18.9; HRMS(FAB) calcd for $C_{20}H_{25}NO_8Cl_3$ [M + $H^+$]; 512.0646, found: 512.0653.

**Compound 5.** Compound **4** (202 mg, 0.40 mmol) and 1H-tetrazole were premixed and coevaporated with toluene and dissolved in 10 mL of $CH_2Cl_2$. The reaction solution was cooled to −30 °C and dibenzyl *N*,*N*-diisopropylphosphamide (0.27 mL, 0.79 mmol) was added. The reaction was warmed to room temperature in 30 min and stirred for another hour. Then the reaction was cooled to −40 °C, and *m*-CPBA (560 mg, 2 mmol) was added. After stirring for 30 min at 0 °C and another 30 min at room temperature, the reaction was diluted with 20 mL of $CH_2Cl_2$, and extracted with 10% aqueous $Na_2SO_3$ (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL), and water (2 × 20 mL). The $CH_2Cl_2$ layer was dried over anhydrous sodium sulfate, filtered, concentrated, and purified by flash chromatograghy (65% EtOAc/petroleum ether) to give 200 mg (70%) of **5** as a white solid: $R_f$ 0.24 (70% EtOAc/petroleum ether); $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 7.44−7.33 (m, 15 H), 7.20 (d, $J$ = 6.0 Hz, 1 H), 6.10 (m, 1 H), 5.56 (s, 1 H), 5.07 (m, 4 H), 5.02 (d, $J$ = 12.0 Hz, 1 H), 4.64 (q, $J$ = 7.0 Hz, 2 H), 4.59 (d, $J$ = 12.0 Hz, 1 H), 4.09 (m, 1 H), 4.03 (m, 1 H), 3.95 (m, 1 H), 3.83−3.68 (m, 3 H), 1.86 (s, 3 H), 1.48 (d, $J$ = 7.0 Hz, 3 H); $^{13}$C NMR ($CDCl_3$, 500 MHz) $\delta$ 173.8, 171.2, 137.1, 129.4, 128.8, 128.5, 128.2, 128.0, 126.1, 101.7, 96.2, 96.1, 82.6, 75.3, 74.3, 74.2, 69.7, 68.6, 64.6, 54.2, 54.1, 23.0, 18.8; HRMS(FAB) calcd for $C_{34}H_{37}NO_{11}Cl_3PNa$ [M + $Na^+$]: 794.1068, found 794.1095.

**Compound 6.** To a solution of compound **5** (58 mg, 0.075 mmol) in 5 mL of 90% AcOH/$H_2O$ was added zinc dust (30 mg). The reaction was stirred vigorously at room temperature for 1 h. The suspension was filtered, and the precipitate was rinsed with methanol. The filtrate was concentrated and purified by flash chromatography (10% MeOH/$CHCl_3$/ 0.1% AcOH) to give 44 mg (91%) of **6** as a white solid: $R_f$ 0.19 (5% MeOH/$CHCl_3$, 0.1% AcOH); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 7.44−7.25 (m, 15 H), 6.11 (m, 1 H), 5.55 (s, 1 H), 5.02 (m, 4 H), 4.33 (q, $J$ = 7.0 Hz, 1 H), 3.96 (m, 1 H), 3.77 (m, 1 H), 3.7−3.66 (m, 4 H), 1.94 (s, 3 H), 1.32 (d, $J$ = 7.0 Hz, 3 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 181.2, 174.2, 139.0, 137.1, 130.0, 129.9, 129.3, 129.2, 127.3, 102.8, 97.4, 83.2, 78.3, 75.0, 71.2, 69.2, 66.4, 56.2, 56.1, 22.8, 19.7; HRMS(FAB) calcd for $C_{32}H_{36}NO_{11}PNa$ [M + $Na^+$]: 664.1924, found 664.1938.

**Compound 7. 1. Fmoc-L-Lys(N-TEOC)-OH.** To a solution of Fmoc-L-Lys(N-BOC)-OH (607 mg, 1.30 mmol) in 10 mL of $CH_2Cl_2$ was added 10 mL of trifluoroacetic acid. The mixture was stirred for 20 min at room temperature and concentrated. The residue was dissolved in 10 mL of DMF. Diisopropylethylamine (1.1 mL, 6.48 mmol) was added. 2-(Trimethylsilyl)ethyl *p*-nitrophenyl carbonate (440 mg, 1.55 mmol) was dissolved in 3 mL of DMF and transferred into the reaction solution. After stirring for 2 h at room temperature, solvent was removed under vacuum. The residue was purified by flash chromatography (eluting first with EtOAc then with 10% MeOH/$CHCl_3$/ 0.1% AcOH) to give 635 mg (95%) of the desired product as a white solid: $R_f$ 0.54 (10% MeOH/$CHCl_3$).

**2. *Z*-D-Glu(OH)-OTMSE.** To a solution of *Z*-D-Glu(O-bzl)-OH (1.1 g, 3.0 mmol) and DMAP (37 mg, 0.3 mmol) in 30 mL of EtOAc was added DCC (0.7 g, 3.6 mmol) and 2-(trimethylsilyl)ethanol (0.5 mL, 3.6 mmol). After stirring for 20 min at room temperature, the reaction was filtered. The filtrate was concentrated and purified by flash chromatography (15% EtOAc/petroleum ether) to give 1.3 g (91%) of *Z*-D-Glu(O-bzl)-OTMSE as a white solid: $R_f$ 0.30 (15% EtOAc/petroleum ether).

To a solution of *Z*-D-Glu(O-bzl)-OTMSE (1.2 g, 2.6 mmol) in 30 mL of MeOH was added 900 mg of 20% Pd−C. After stirring for 10 min at room temperature, the suspension was filtered. The filtrate was concentrated and dissolved in 20 mL of $H_2O$/dioxane (1:1). To the solution was added $NaHCO_3$ (0.44, 5.2 mmol). A solution of Cbz-succinimide (0.8 g, 3.1 mmol) in 5 mL of dioxane was added to the reaction over 30 min. Then 1 mL of AcOH was added. Solvent was removed under vacuum. The residue was purified by flash chromatography (eluting first with 10% EtOAc/$CH_2Cl_2$ then with 10% MeOH/$CHCl_3$/0.1% AcOH) to give 0.9 g (87%) of *Z*-D-Glu(OH)-OTMSE as a white solid: $R_f$ 0.49 (10% MeOH/$CHCl_3$); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 7.24−7.15 (m, 5 H), 4.96 (d, $J$ = 3.0 Hz, 1 H), 4.10 (m, 4 H), 2.28 (t, $J$ = 7.6 Hz, 2 H), 2.02 (m, 1 H), 1.80 (m, 1 H), 0.87 (t, $J$ = 8.6 Hz, 2 H), −0.08 (s, 9 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 176.3, 173.9, 158.6, 138.2, 129.5, 129.1, 128.9, 67.7, 64.7, 55.0, 31.2, 27.8, 18.2, −1.3;

Peptide **7** was synthesized by standard HOBt/HBTU method with Fmoc-protected amino acids. $R_f$ 0.29 (10% MeOH/$CHCl_3$); $^1$H NMR (DMSO, 500 MHz) $\delta$ 8.15 (d, $J$ = 5.0 Hz, 1 H), 8.14 (d, $J$ = 5.0 Hz, 1 H), 8.10 (d, $J$ = 8.0 Hz, 1 H), 8.02 (d, $J$ = 5.0 Hz, 1 H), 6.92 (t, $J$ = 5.0 Hz, 1 H), 4.30 (m, 1 H), 4.19 (m, 2 H), 4.17−4.07 (m, 5 H), 4.00 (t, $J$ = 8.5 Hz, 2 H), 3.31 (q, $J$ = 8.5 Hz, 1 H), 2.92 (m, 2 H),

2.18 (m, 2 H), 1.95 (m, 1 H), 1.80 (m, 1 H), 1.57 (m, 1 H), 1.48 (m, 1 H), 1.36 (m, 2 H), 1.29 (d, $J = 8.5$ Hz, 3 H), 1.25 (m, 2 H), 1.20 (d, $J = 8.5$ Hz, 3 H), 1.13 (d, $J = 8.5$ Hz, 3 H), 0.92 (m, 6 H), 0.02−0.00 (3s, 27 H); $^{13}$C NMR (DMSO, 500 MHz) $\delta$ 175.8, 172.3, 172.0, 171.8, 171.5, 171.4, 156.2, 62.6, 62.4, 61.2, 52.9, 51.3, 50.1, 47.7, 47.6, 31.4, 31.2, 29.2, 27.2, 22.6, 21.4, 18.0, 17.4, 16.9, 16.8, 16.7, −1.4, −1.5, −1.6; HRMS(FAB) calcd for $C_{36}H_{72}N_6O_{10}Si_3Na$ [M + $Na^+$]: 855.4515, found: 855.4564.

**Compound 8.** To a solution of compound **6** (85 mg, 0.13 mmol) and $NH_2$-L-Ala-$\gamma$-D-Glu(O-TMSE)-L-Lys(N-TEOC)-D-Ala-D-Ala-OTMSE (**7**) (153 mg, 0.18 mmol) in 1.5 mL of DMF was added diisopropylethylamine (116 mL, 0.66 mmol) followed by HOBt (27 mg, 0.20 mmol) and PyBOP (104 mg, 0.20 mmol). After stirring for 30 min at room temperature, the solution was diluted in 10 mL of EtOAc and washed with 0.01 N aqueous HCl (3 × 10 mL). The organic layer was concentrated, dried over anhydrous sodium sulfate, and purified by flash chromatograghy (5% MeOH/$CHCl_3$) to give 168 mg (87%) of **8** as a white solid: $R_f$ 0.24 (5% MeOH/$CHCl_3$); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 7.52−7.37 (m, 15 H), 5.88 (m, 1 H), 5.65 (s, 1 H), 5.13 (m, 4 H), 4.41 (m, 2 H), 4.35 (m, 3 H), 4.17 (m, 8 H), 4.06 (dd, $J = 9.5$, 3.5 Hz, 1 H), 3.84 (m, 3 H), 3.77 (m, 1 H), 3.10 (m, 2 H), 2.29 (t, $J = 14.5$ Hz, 2 H), 2.19 (m, 1 H), 1.90 (m, 1 H), 1.88 (s, 3 H), 1.77 (m, 1 H), 1.67 (m, 1 H), 1.51 (m, 2 H), 1.43−1.35 (m, 14 H), 1.01−0.97 (m, 6 H), 0.06−0.04 (3s, 27 H); $^{13}$C NMR ($CDCl_3$, 500 MHz) $\delta$ 173.9, 172.8, 172.4, 171.8, 171.3, 157.1, 137.1, 135.5, 135.4, 129.2, 129.0, 128.9, 128.7, 128.4, 128.1, 126.1, 101.6, 97.1, 82.5, 81.0, 78.2, 76.7, 70.0, 69.6, 68.4, 64.8, 64.1, 63.8, 63.0, 53.9, 53.3, 51.4, 50.0, 49.1, 48.4, 40.4, 31.6, 31.5, 29.6, 27.9, 23.1, 22.7, 19.6, 18.0, 17.9, 17.8, 17.5, 17.4, −1.3, −1.4, −1.5; HRMS(FAB) calcd for $C_{68}H_{106}N_7O_{20}PSi_3Na$ [M + $Na^+$]: 1478.6436, found: 1478.6417.

**Compound 9.** To a solution of compound **8** (87 mg, 0.06 mmol) in 5 mL of MeOH was added 20 mg of 20% Pd−C. The reaction vessel was filled with hydrogen and stirred at room temperature. After 30 min 1 mL of pyridine was added. The solution was diluted with 15 mL of MeOH and stirred for 30 min. The catalyst was filtered off. The filtrate was concentrated to give product **9** which was used in the next reaction without further purification. $R_f$ 0.28 ($CHCl_3$:MeOH:$H_2O$ = 3:2:0.5).

**Compound 11**. Compound **9** (58 mg, 0.04 mmol) was coevaporated with toluene (3 × 1 mL) and dissolved in 1 mL of $CH_2Cl_2$. A portion of the citronellol diphenylpyrophosphate (**10**) solution (0.4 mL) was added to the reaction followed by an addition of pyridine (20 $\mu$L, 0.24 mmol). The reaction was stirred at room temperature for 18 h. Solvent was removed under vacuum and the residue was loaded to a C18 reverse phase column (8 mm × 80 mm, particle size 40 mm, pore size 60 Å, from J. T. Baker) and eluted with $CH_3CN$/0.1% $NH_4HCO_3$ aqueous solution (0, 5%, 10%, 15%, 20%, 25%, 30%, 35% of 10 mL each). The fractions containing desired product were combined and concentrated to give 34 mg (68%) of **11** as a white powder. $R_f$ 0.21 ($CHCl_3$:MeOH:$H_2O$ = 4.5:1.5:0.2). This product was used in the next reaction without further purification. ESI-MS calcd for $C_{57}H_{109}N_7O_{23}P_2Si_3Na$ [M + $Na^+$]: 1429, found: 1429.

**Compound 1a.** To a solution of compound **11** (43 mg, 0.023 mmol) in 0.7 mL of DMF was added tetrabutylammonium fluoride (1 M in THF, 0.7 mL). The reaction was stirred at room temperature for 24 h. Solvent was removed under vacuum. The residue was loaded to a C18 reverse phase column (8 mm × 80 mm, particle size 40 $\mu$m, pore size 60 Å, from J. T. Baker), and eluted with $CH_3CN$/0.1% $NH_4HCO_3$ aqueous solution (0, 5%, 10%, 15%, 20%, 25%, 30% of 10 mL each). The fractions containing the desired product were combined and concentrated. The crude product was further purified on a diethylaminoethyl cellulose column (14 mm × 80 mm, from Whatman Labsales, Inc.), eluted with 250 mM $NH_4HCO_3$, to give 24 mg of **1a** (93%) as a white powder after lyophilization: $R_f$ 0.18 ($CHCl_3$:MeOH: $H_2O$ = 3:3:1); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 5.58 (m, 1 H), 5.11 (t, $J = 6.5$ Hz, 1 H), 4.50−3.56 (m, 12 H), 2.94 (m, 2 H), 2.34 (m, 2 H), 2.10 (s, 3 H), 2.00 (m, 1 H), 1.98 (m, 2 H), 1.92 (m, 1 H), 1.74 (m, 2 H), 1.67 (s, 3 H), 1.62 (m, 1 H), 1.60 (s, 3 H), 1.50−1.39 (m, 12 H), 1.23 (m, 2 H), 0.93 (d, $J = 6.5$ Hz, 3 H); $^{13}$C NMR ($D_2O$, 500 MHz) $\delta$ 178.2, 177.9, 176.7, 176.6, 176.5, 176.4, 176.3, 165.3, 135.5, 127.6, 97.0, 82.2, 80.3, 75.4, 74.1, 72.0, 71.9, 71.8, 70.4, 67.6, 62.7, 56.6, 55.8, 52.3, 51.9, 51.2, 41.5, 38.8, 34.0, 32.7, 31.0, 30.0, 28.6, 27.2, 27.1, 24.6, 24.4, 21.0, 19.2, 19.1, 18.8; ESI-MS calcd for $C_{41}H_{74}O_{21}N_7P_2$ [M + $H^+$]: 1062, found: 1062.

**Compound 1b.** To a solution of compound **1a** (25 mg, 0.022 mmol) in 1.5 mL of $H_2O$/dioxane(1:1) was added $NaHCO_3$ (23 mg, 0.4 mmol) followed by 6-((biotinoyl)amino)hexanoic acid succinimide ester (12 mg, 0.027 mmol). The reaction was stirred at room temperature for 2 h. Solvent was removed under vacuum. The residue was loaded on a diethylaminoethyl cellulose column (14 mm × 80 mm, from Whatman Labsales, Inc.), eluted with 250 mM $NH_4HCO_3$ to give 16 mg (80%) of **1b** as a white powder after lyophilization. $R_f$ 0.40 ($CHCl_3$:MeOH:$H_2O$ = 3:3:1); $^1$H NMR ($CD_3OD$, 500 MHz) d 5.49 (dd, $J = 3.0$, 7.3 Hz, 1 H), 5.11 (t, $J = 7.2$ Hz, 1 H), 4.50 (dd, $J = 4.8$, 7.8 Hz, 1 H), 4.37 (m, 2 H), 4.31 (dd, $J = 4.3$, 7.8 Hz, 1 H), 4.29 (m, 1 H), 4.24 (m, 3 H), 4.16 (d, $J = 10.4$ Hz, 1 H), 4.02 (m, 2 H), 3.99 (m, 1 H), 3.90 (d, $J = 11.0$ Hz, 1 H), 3.74 (m, 1 H), 3.70 (m, 1 H), 3.49 (dd, $J = 9.5$, 9.5 Hz, 1 H), 3.21 (m, 1 H), 3.17 (m, 4 H), 2.94 (dd, $J = 4.8$, 12.8 Hz, 1 H), 2.71 (d, $J = 12.8$ Hz, 1 H), 2.31 (m, 1 H), 2.28 (m, 2 H), 2.25 (m, 1 H), 2.20 (m, 4 H), 2.02 (s, 3 H), 2.00 (m, 2 H), 1.86 (m, 2 H), 1.82 (m, 1 H), 1.73 (m, 4 H), 1.67 (s, 3 H), 1.63 (m, 5 H), 1.61 (s, 3 H), 1.52 (m, 4 H), 1.45 (m, 2 H), 1.44 (d, $J = 7.3$ Hz, 3 H), 1.43 (d, $J = 6.2$ Hz, 3 H), 1.41 (m, 2 H), 1.38 (d, $J = 7.3$ Hz, 3 H), 1.37 (d, $J = 7.2$ Hz, 3 H), 1.35 (m, 2 H), 1.17 (m, 1 H), 0.93 (d, $J = 6.7$ Hz, 3 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 177.2, 176.5, 176.2, 176.1, 176.0, 175.6, 174.7, 174.6, 174.5, 174.2, 166.3, 132.1, 126.2, 96.4, 81.3, 78.8, 75.2, 71.0, 65.7, 63.6, 63.0, 61.8, 57.2, 55.7, 55.0, 54.2, 50.9, 50.7, 50.4, 41.2, 40.4, 40.2, 39.1, 39.0, 38.6, 37.2, 37.0, 33.0, 32.5, 30.6, 30.3, 30.2, 30.0, 29.6, 27.7, 27.1, 26.9, 26.7, 26.1, 24.5, 23.5, 20.0, 19.5, 18.4, 18.3, 18.0, 17.9; HRMS(FAB) calcd for $C_{57}H_{95}N_{10}O_{24}P_2SNa_2$ [M-3$H^+$+2$Na^+$]: 1443.5512, found: 1443.5494.

**Compound 12a.** Compound **9** was deprotected with TBAF using the same method for making **1a**. $R_f$ 0.16 ($CHCl_3$:MeOH:$H_2O$ = 3:4:1.5); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 5.34 (dd, $J = 3.0$, 7.0 Hz, 1 H), 4.24 (m, 3 H), 4.17 (dd, $J = 6.7$, 6.7 Hz, 1 H), 4.08 (dd, $J = 4.6$, 8.5 Hz, 1 H), 4.03 (q, $J = 7.0$ Hz, 1 H), 3.93 (m, 1 H), 3.80 (m, 1 H), 3.75 (m, 1 H), 3.59 (dd, $J = 5.5$, 11.6 Hz, 1 H), 3.56 (m, 1 H), 3.38 (dd, $J = 9.7$, 9.7 Hz, 1 H), 2.82 (t, $J = 7.3$ Hz, 2 H), 2.22 (m, 2 H), 2.15 (m, 1 H), 1.86 (s, 3 H), 1.70 (m, 4 H), 1.58 (m, 2 H), 1.40 (m, 1 H), 1.31 (m, 6 H), 1.25 (m, 6 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 179.4, 178.8, 178.0, 176.2, 175.9, 174.7, 174.0, 173.8, 95.3, 81.2, 78.7, 74.9, 71.2, 62.8, 55.5, 55.3, 55.0, 51.9, 51.1, 50.8, 40.5, 33.1, 32.5, 30.4, 28.4, 23.7, 23.4, 19.8, 19.4, 18.4, 18.0; HRMS(FAB) calcd for $C_{31}H_{53}N_7O_{18}P$ [M − $H^+$]: 842.3185, found: 842.3212.

**Compound 12b.** Compound **12b** was made from **12a** and 6-((biotinoyl)amino)hexanoic acid succinimide ester using the same chemistry described in step i (Scheme 2). $R_f$ 0.27 ($CHCl_3$:MeOH:$H_2O$ = 3:4:1.5); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 5.45 (dd, $J = 7.0$, 3.0 Hz, 1 H), 4.51 (dd, $J = 5.0$, 7.5 Hz, 1 H), 4.39 (m, 2 H), 4.32 (m, 2 H), 4.26 (m, 3 H), 4.12 (m, 1 H), 3.91 (m, 1 H), 3.86 (d, $J = 11.6$ Hz, 1 H), 3.73 (dd, $J = 5.5$, 11.6 Hz, 1 H), 3.69 (m, 1 H), 3.53 (m, 1 H), 3.22 (m, 1 H), 3.17 (m, 4 H), 2.94 (dd, $J = 5.0$, 12.8 Hz, 1 H), 2.72 (d, $J = 12.8$ Hz, 1 H), 2.30 (m, 4 H), 2.21 (m, 4 H), 1.99 (s, 3 H), 1.89 (m, 1 H), 1.82 (m, 1 H), 1.74 (m, 2 H), 1.63 (m, 4 H), 1.53 (m, 4 H), 1.46 (m, 2 H), 1.44 (m, 6 H), 1.39 (m, 6 H), 1.35 (m, 4 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 177.5, 177.4, 177.3, 176.5, 176.3, 174.8, 174.7, 174.6, 174.3, 174.2, 166.0, 94.3, 80.6, 78.6, 73.2, 68.9, 62.8, 61.1, 60.9, 56.1, 55.0, 54.3, 54.2, 51.6, 50.4, 50.1, 40.4, 39.8, 39.6, 36.4, 36.2, 32.5, 31.4, 28.8, 28.7, 28.6, 28.5, 28.4, 26.2, 25.9, 25.8, 23.2, 22.7; HRMS-(FAB) calcd for $C_{47}H_{78}N_{10}O_{21}PS$ [M − $H^+$]: 1181.4801, found: 1181.4769.

**Compound 13.** Compound **13** was made following the same scheme as **1a** except that in step e, intermediate **6** was coupled to dipeptide $CH_3NH$-D-$\gamma$-Glu(O-TMSE)-L-Ala-$NH_2$ instead of to **7**. $R_f$ 0.41 ($CHCl_3$:MeOH:$H_2O$ = 3:3:1); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 5.49 (dd, $J = 3.0$, 7.0 Hz, 1 H), 5.11 (t, $J = 6.6$ Hz, 1 H), 4.33 (q, $J = 7.0$ Hz, 1 H), 4.27 (q, $J = 7.0$ Hz, 1 H), 4.24 (dd, $J = 3.8$, 7.6 Hz, 1 H), 4.16 (m, 1 H), 4.04 (m, 2 H), 4.00 (m, 1 H), 3.90 (dd, $J = 1.8$, 11.8 Hz, 1 H), 3.75 (dd, $J = 9.6$, 9.6 Hz, 1 H), 3.70 (dd, $J = 5.7$, 11.8 Hz, 1 H), 3.48 (dd, $J = 9.6$, 9.6 Hz, 1 H), 2.64 (s, 3 H), 2.18 (m, 2 H), 2.16 (m, 1 H), 2.02 (s, 3 H), 1.98 (m, 2 H), 1.92 (m, 1 H), 1.72 (m, 1 H), 1.67 (s, 3 H), 1.62 (m, 1 H), 1.61 (s, 3 H), 1.47 (m, 1 H), 1.43 (d, d, $J = 7.0$ Hz,

6 H), 1.37 (m, 1 H), 1.18 (m, 1 H), 0.94 (d, $J = 6.6$ Hz, 3 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 177.2, 176.1, 176.0, 174.4, 174.2, 132.0, 126.1, 96.3, 81.1, 78.9, 75.2, 70.8, 65.7, 63.0, 55.1, 54.9, 51.0, 39.1, 38.6, 33.3, 30.6, 30.1, 26.7, 26.5, 26.1, 23.4, 19.9, 19.5, 18.2, 17.9; HRMS(FAB) calcd for $C_{30}H_{53}N_4O_{17}P_2$ [M − $H^+$]: 803.2881, found: 803.2861.

**Compound 14a.** Compound **14a** was made following the same scheme as **1a** except that in step e, compound **6** was coupled to TEOC-$NHCH_2CH_2NH_2$ instead of to **7**. The sily-protecting group was cleaved using TBAF, the same as in making **1a**. $R_f$ 0.20 ($CHCl_3$:MeOH:$H_2O$ = 3:2:0.5); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 5.58 (bs, 1 H), 5.11 (t, $J$ = 7.0 Hz, 1 H), 4.30 (q, $J = 6.7$ Hz, 1 H), 4.21 (m, 1 H), 4.04 (m, 3 H), 3.72 (m, 1 H), 3.78 (m, 1 H), 3.73 (m, 1 H), 3.64 (m, 1 H), 3.50 (dd, $J = 9.4$, 9.4 Hz, 1 H), 3.40 (m, 1 H), 3.13 (m, 2 H), 2.03 (s, 3 H), 2.00 (m, 2 H), 1.73 (m, 1 H), 1.67 (s, 3 H), 1.63 (m, 1 H), 1.61 (s, 3 H), 1.46 (m, 1 H), 1.39 (m, 1 H), 1.38 (d, $J = 6.7$ Hz, 3 H), 1.18 (m, 1 H), 0.94 (d, $J = 6.7$ Hz, 3 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 176.2, 173.6, 131.2, 125.2, 95.6, 80.7, 78.1, 74.3, 70.3, 64.9, 62.0, 54.2, 39.7, 38.2, 37.8, 37.5, 29.8, 25.8, 25.2, 22.5, 19.1, 18.6, 17.0; HRMS-(FAB) calcd for $C_{23}H_{43}N_3O_{13}P_2Na$ [M − $2H^+$ + $Na^+$]: 654.2169, found 654.2199.

**Compound 14b.** Compound **14b** was made from **14a** and 6-((biotinoyl)amino)hexanoic acid succinimide ester using the same chemistry described in step i (Scheme 2): $R_f$ 0.30 ($CHCl_3$:MeOH:$H_2O$ = 3:2:0.5); $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$ 5.49 (dd, $J = 2.7$, 7.0 Hz, 1 H), 5.12 (t, $J = 7.2$ Hz, 1 H), 4.50 (dd, $J = 5.0$, 7.5 Hz, 1 H), 4.32 (dd, $J$ = 4.4, 7.5 Hz, 1 H), 4.20 (q, $J = 6.7$ Hz, 1 H), 4.16 (m, 1 H), 4.03 (m, 2 H), 3.98 (m, 1 H), 3.90 (d, $J = 12.0$ Hz, 1 H), 3.71 (m, 1 H), 3.70 (m, 1 H), 3.45 (dd, $J = 9.4$, 9.4 Hz, 1 H), 3.23 (m, 1 H), 3.18 (m, 6 H), 2.94 (dd, $J = 5.0$, 12.8 Hz, 1 H), 2.72 (d, $J = 12.8$ Hz, 1 H), 2.24 (t, $J = 7.6$ Hz, 2 H), 2.21 (t, $J = 7.6$ Hz, 2 H), 2.03 (s, 3 H), 2.00 (m, 2 H), 1.73 (m, 3 H), 1.68 (s, 3 H), 1.64 (m, 6 H), 1.62 (s, 3 H), 1.53 (m, 2 H), 1.45 (m, 3 H), 1.40 (d, $J = 6.7$ Hz, 3 H), 1.36 (m, 3 H), 1.18 (m, 1 H), 0.94 (d, $J = 6.7$ Hz, 3 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 176.5, 176.4, 176.1, 174.4, 166.3, 132.0, 126.1, 96.5, 81.9, 79.2, 75.2, 70.9, 65.7, 63.5, 62.9, 61.8, 57.1, 55.0, 41.2, 40.4, 40.1, 39.1, 39.0, 38.6, 37.2, 37.0, 30.6, 30.3, 30.0, 29.6, 27.8, 27.1, 26.8, 26.7, 26.1, 23.4, 20.0, 19.6, 18.0; HRMS(FAB) calcd for $C_{39}H_{69}N_6O_{16}P_2S$ [M − $H^+$]: 971.3966, found: 971.3948.

**Compound 15.** To a solution of **2** (20 mg, 0.042 mmol) in 1 mL of $CH_2Cl_2$ was added DIEA (16 $\mu$L, 0.924 mmol). The reaction vessel was cooled to −30 °C, then MeOTf (5.2 $\mu$L, 0.046 mmol) was added. The reaction was complete after stirring at room temperature for 30 min. Saturated $NaHCO_3$ was added. The mixture was extracted with $CH_2Cl_2$ (3 × 5 mL). The organic layers were combined, dried over anhydrous sodium sulfate, filtered, concentrated, and purified by flash chromatography (45% EtOAc/petroleum ether) to give 18 mg (87%) of product as a white powder. The following chemistry was the same as in Scheme 2: $R_f$ 0.12 ($CHCl_3$:MeOH:$H_2O$ = 3:2:0.5); $^1$H NMR ($CD_3$-OD, 500 MHz) $\delta$ 5.50 (dd, $J = 3.4$, 7.3 Hz, 1 H), 4.58 (q, $J = 6.7$ Hz, 1 H), 3.87 (m, 2 H), 3.84 (m, 1 H), 3.73 (3, 3 H), 3.62 (m, 2 H), 3.42 (dd, $J = 9.2$, 9.2 Hz, 1 H), 2.00 (s, 3 H), 1.37 (d, $J = 7.0$ Hz, 3 H); $^{13}$C NMR ($CD_3OD$, 500 MHz) $\delta$ 176.4, 173.8, 95.0, 80.7, 77.3, 74.8, 72.8, 62.9, 54.9, 52.6, 23.2, 19.4; ESI-MS calcd for $C_{12}H_{23}NO_{11}P$ [M + $H^+$]: 388, found: 388.

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