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Synthesis and Biological Evaluation of Analogues of Bacterial Lipid I

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Abstract—Bacterial Lipid I analogues containing different anomeric groups at the muramic acid moiety were synthesized and screened in MurG enzyme assays run in the presence and absence of cell wall membranes. The results obtained in this study help elucidate the role of the lipid diphosphate in the recognition of Lipid I by MurG.

The available arsenal of antibiotics was once thought to be powerful enough to fight most bacterial infections. However, identification of deadly multiple drug resistant bacterial strains in the last decades has created the need for novel, more potent antibiotics. One of the prime targets for drug discovery is cell wall biosynthesis, an essential process in the bacterial life cycle.[1,2] Unfortunately, enzymatic steps that take place late in the pathway have not been fully characterized to date, mainly because the enzymes are either membrane-associated or membrane-bound, complicating isolation and biochemical studies.[3] Furthermore, the substrates for these enzymes are not readily isolated because of low abundance in cellular systems and unfavorable physical properties.[4] Such problems must be overcome in order to allow a clearer understanding of the enzymatic processes and the discovery of novel enzyme inhibitors for this pathway.

MurG (UDP-*N*-acetylglucosamine: undecaprenyl-pyrophosphoryl-*N*-acetylmuramoyl-pentapeptide *N*-acetyl glucosaminyl transferase) [GenBank P17443] is a cytoplasmatic membrane-associated GlcNAc transferase, responsible for the conversion of Lipid I (**1**) to Lipid II (**2**), the substrate for transglycosylases and transpeptidases.[5] Although the MurG enzyme was first described in 1965, studies of its mechanism have lagged because Lipid I, the sugar acceptor of MurG, could not be isolated in large amounts from natural sources. In 1998 Walker et al. showed that **3**, a simpler version of Lipid I with a short citronellol lipid (Fig. 2), was efficiently processed by MurG in solution.[6] This readily accessible substrate allowed the authors to study the kinetic properties of the enzyme in the absence of membranes. Walker et al. evaluated the ability of phosphorylated Lipid I analogues incorporating changes in the lipid and peptide portions to act as alternative substrates or dead-end inhibitors for MurG.[7] In these studies the anomeric phosphate (**4**) inhibited MurG with an IC_{50} of ($50\pm25\,\mu g\ mL^{-1}$), but had no acceptor ability, suggesting that the anomeric group in Lipid I played a key role in acceptor recognition.

We have recently described a coupled MraY/MurG assay, designed to identify and characterize novel inhibitors of these enzymes.[8] This assay uses biotinylated UDP-MurNAc-pentapeptide and UDP-[^{14}C]-GlcNAc as substrates. The assay utilizes *E. coli* cell wall membrane fragments as enzyme source, and is based on the specific capture of biotinylated Lipid II by avidin-coated beads. This coupled MraY/MurG assay differs considerably from the assay developed by Walker et al. since the latter uses **3** as the substrate for soluble MurG in the *absence* of membranes, circumventing issues related to solution-membrane partition of the substrate. In an effort to obtain more detailed information about the substrate requirements of MurG, we decided to study the behavior of analogues of Lipid I in the MraY/MurG assay. In these compounds the anomeric diphosphoryl lipid unit was replaced by a simple phosphate group (**4**)

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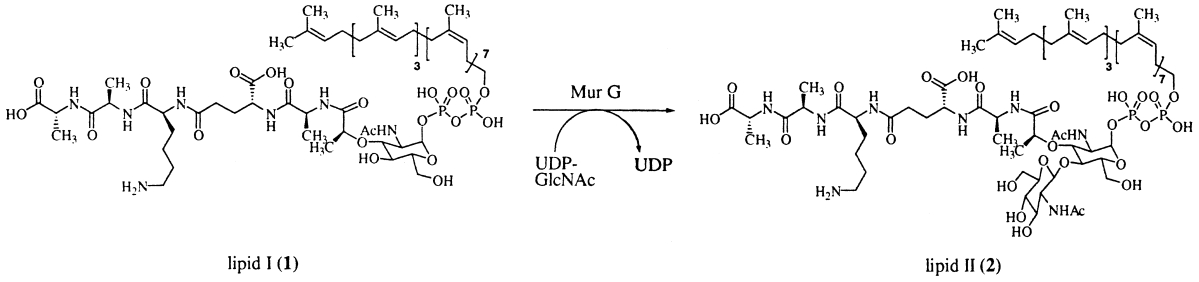

Figure 1.

or noncharged groups, such as the methoxy group (**5a**) and the polarizable hydrophobic thiophenoxy group (**5b**).[9] Screening of these compounds in MurG assays should potentially shed further light on the role of the lipid diphosphate moiety in acceptor recognition and glycosylation, and help establish the mechanism of MurG catalysis.

Lipid I derivatives **5a**–**b** were prepared by synthesizing the carbohydrate and peptide moieties separately, coupling them through an amide linkage and performing final deprotection. The protected muramic acids were synthesized from the common intermediate **6** (Fig. 3), obtained in three steps from glucosamine hydrochloride.

Compounds **6** and **7** (prepared from **6** via the reactive β-bromide) were deacetylated, protected as 4,6-benzylidene acetals and alkylated with methyl-(1*S*)-*O*-trifluoromethanesulfonyl lactate.[10] The corresponding esters **9a**–**b** were saponified and neutralized, yielding the muramic acids **10a**–**b**. Pentapeptide **11** (Fig. 4) was synthesized from commercially available *N*-Boc protected amino acids, using a sequence of HATU-promoted amide couplings and TFA acidic deprotections.

Muramic acids **10a**–**b** were coupled to the pentapeptide **11** using HATU (Fig. 4). Glycopeptides **12a**–**b** were reduced to the free amines with PMe_3 and then acetylated. The benzylidene groups were selectively removed using TFA in CH_3CN–$CHCl_3$ to produce diols **13a**–**b**. Finally, treatment with 0.1 M LiOH in THF–MeOH–H_2O removed all base sensitive protective groups to yield final products **5a**[11] and **5b**.[12]

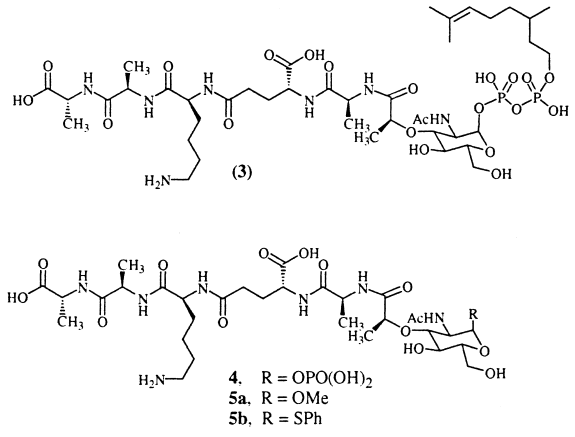

Figure 2. Lipid I analogues.

Lipid analogues **5a** and **5b** were tested in an assay modeled after Walker et al. using **3** as substrate and either purified soluble MurG or *E. coli* membranes as enzyme source. In either case, no inhibition of MurG was detected even at 200 μg mL^{-1} of the compounds.

Analogues **4**, **5a**, and **5b** were tested in the coupled MraY/MurG assay for their ability to inhibit the activity of either enzyme. The analogues were tested at concentrations up to 200 μg mL^{-1} (238, 256, and 234 μM, respectively), but no inhibition of Lipid II synthesis was observed. Under the same assay conditions, tunicamycin[13] and ramoplanin[14] (known inhibitors of MraY and MurG, respectively) had IC_{50} values lower than 1 μg mL^{-1}.

Lipid I analogues **4**, **5a**, and **5b** were further tested in a peptidoglycan formation assay, which measures the formation of peptidoglycan by assessing incorporation of ^{14}C-GlcNAc (contained in Lipid II) into TCA-insoluble, filter-capturable material.[15] Once again, no inhibition was observed for compound **4**, **5a**, or **5b** at concentrations of 200 μg mL^{-1},[16] suggesting that these analogues do not affect downstream processing of Lipid II to peptidoglycan polymer.

These results provide interesting insights into the role of the lipid diphosphate in MurG activity. Whereas **4** behaves as an inhibitor for MurG in the soluble enzyme assay,[7] this compound was found to be inactive in the coupled MraY/MurG assay, suggesting that **4** cannot compete efficiently for the active site of *membrane-bound* MurG. Compounds **5a** and **5b** were inactive in both

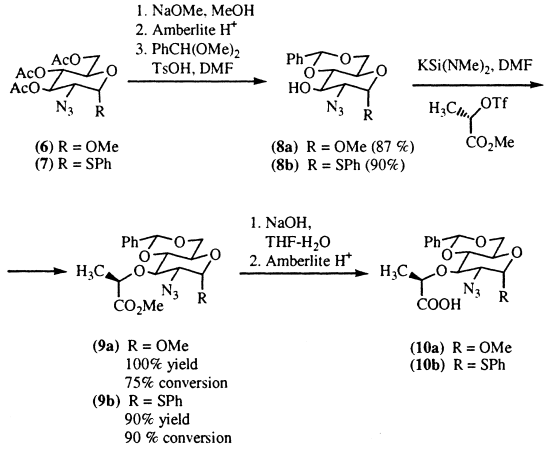

Figure 3. Synthesis of protected muramic acids.

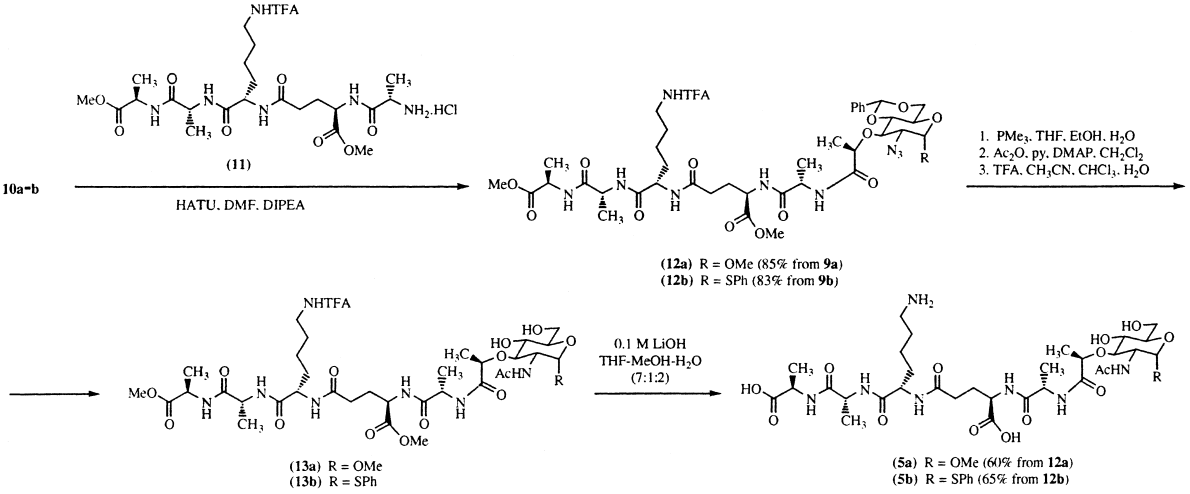

Figure 4. Coupling and deprotection of analogues **5a** and **5b**.

assays. Taken together, our studies indicate that the anomeric substituent of the muramic acid in Lipid I plays a key role in substrate recognition and processing. The lipid phosphate may not only help determine the substrate specificity of the MurG enzyme (**1** and **3** are substrates, **4** is an inhibitor, and **5a** and **5b** are inactive) but also may localize the Lipid I substrate into the cell membrane, avoiding the processing of other possible solution-based substrates or inhibitors (**4** is an inhibitor in the solution assay but not in the membrane-based coupled assay). The chemistry described here allowed us to synthesize the desired Lipid I analogues in an efficient and convergent manner. The synthetic route to these glycopeptides is flexible and can be extended to the synthesis of Lipid I derivatives containing different substituents at the anomeric center. Present work concentrates on the synthesis of lipid analogues containing pyrophosphate mimics at the anomeric center of the muramic acid and testing of these compounds in the soluble MurG and coupled MraY/MurG assays.

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9. Compound **4** was obtained as a kind gift from Prof. Suzanne Walker (Princeton University).
10. (a) Synthesis of the alkylating reagent was adapted from: Vedejs, E.; Engler, D. A.; Mullins, M. J. *J. Org. Chem.* **1977**, *42*, 3109. (b) Alkylation procedure was adapted from: Kinzy, W.; Schmidt, R. R. *Liebigs. Ann. Chem.* **1987**, 407.
11. Analytical data for **5a**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.43–7.40 (m, 2H), 7.28–7.20 (m, 3H), 5.81 (d, 1H, $J=5.4$ Hz), 4.60 (q_{app}, 1H, $J=6.6$ Hz), 4.43 (q_{app}, 1H, $J=7.5$ Hz), 4.35–4.30 (m, 2H), 4.17 (br, 2H), 4.02 (m, 2H), 3.77 (m, 2H), 3.59 (m, 2H), 2.89 (br, 2H), 2.33 (br, 3H), 1.92 (s, 3H), 1.90–1.80 (m, 3H), 1.63 (br, 2H), 1.50–1.30 (m, 14H); ^{13}C NMR (75.4 MHz, CD_3OD) δ 176.65, 174.22 (br), 173.60, 135.75, 133.13, 130.06, 128.46, 88.96, 79.88, 77.63, 75.23, 72.11, 62.21, 55.78, 54.99, 50.80, 44.94, 40.48, 33.07, 32.21, 29.60, 28.16, 23.49, 22.91, 20.18, 18.66, 18.20, 17.93. MS (ES) m/z obsd 856 $[M+H^+]$.
12. Analytical data for **5b**: ^{1}H NMR (300 MHz, CD_3OD) δ 4.68 (d, 1H, $J=3.6$ Hz), 4.37–4.31 (m, 6H), 3.97 (dd, 1H, $J=3.0$ Hz, 10.2 Hz), 3.80 (t, 1H, $J=11.4$ Hz), 3.71 (dd, 1H, $J=4.8$ Hz, 12.3 Hz), 3.63–3.45 (m, 3H), 3.35 (s, 3H), 2.94 (br, 2H), 2.32 (br, 3H), 1.94 (s, 3H), 2.00–1.82 (m, br, 3H), 1.68 (br, 2H), 1.51–1.30 (m, 14H); ^{13}C NMR (75.4 MHz, CD_3OD) δ 176.14, 175.47, 174.27, 174.15, 173.36, 99.73, 80.80, 78.31, 73.82, 71.21, 62.53, 55.52, 55.07, 54.86, 50.80, 50.10, 40.48, 32.95, 32.24, 29.63, 28.15, 23.46, 22.91, 19.75, 18.62, 18.22, 17.90. MS (ES) m/z obsd 777 $[M+H^+]$.
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16. Under these conditions the drug moenomycin, a known transglycosylase inhibitor, had an IC_{50} of 0.004 μg mL^{-1}.