Substrate Synthesis and Activity Assay for MurG

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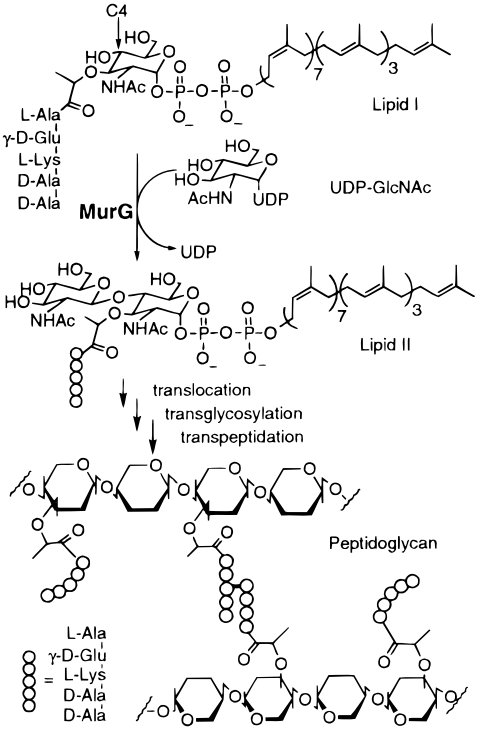
Some of the best antibiotics function by interfering with the biosynthesis of the peptidoglycan polymer that surrounds bacterial cells. With the emergence of bacterial pathogens that are resistant to common antibiotics it has become imperative to learn more about the enzymes involved in peptidoglycan biosynthesis. Although remarkable progress has been made in characterizing some of the early enzymes in the biosynthetic pathway,[1] the downstream enzymes have proven exceedingly difficult to study. This is partly because the downstream enzymes are membrane-associated,[2] making them intrinsically hard to handle, and partly because substrates for many of the enzymes are not readily available.[3] These problems have impeded the development of activity assays suitable for detailed mechanistic investigations of the downstream enzymes.[4]

MurG, a cytoplasmic membrane-associated enzyme, catalyzes the transfer of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to the C4 hydroxyl of an undecaprenyl pyrophosphate *N*-acetylmuramyl pentapeptide substrate (lipid I), assembling the disaccharide−pentapeptide building block (lipid II) which is incorporated into polymeric peptidoglycan (Scheme 1).[5] No mammalian homologues of this enzyme have been identified, and the muramyl pentapeptide substrate is unique to bacteria. These features suggest that it will be possible to design specific MurG inhibitors. However, despite decades of effort spent characterizing MurG activity, there is virtually no structural or mechanistic information on the enzyme.[6] Difficulties isolating lipid I have prevented the development of a simple, direct assay for MurG activity.[3,7] In this paper we report the synthesis of a substrate for MurG and demonstrate its use in an activity assay.

Scheme 1. The Reaction Catalyzed by MurG in the Context of Peptidoglycan Biosynthesis[a]



[a] The composition of the pentapeptide varies with microorganism, particularly at the third position (shown here as L-Lys).[13]

Our first synthetic target, **5a** (Scheme 2), differs from lipid I in that the 55-carbon undecaprenol chain has been replaced by the 10-carbon chain of citronellol. A shorter lipid chain was chosen because long-chain lipids are difficult to handle; a lipid containing a saturated isoprenol unit was chosen because allylic pyrophosphates are unstable. Although MurG is a membrane-associated enzyme which recognizes a lipid-linked substrate, the chemistry takes place on the C4 hydroxyl of the lipid-linked substrate, which is far away from the lipid anchor; therefore, we anticipated that it would be possible to alter the lipid without destroying substrate recognition. To make **5a** (Scheme 2), muramic acid derivative **1** (Sigma) was converted to the anomeric dibenzyl phosphate **2** in five steps and coupled to the protected pentapeptide **3**.[8,9] Hydrogenolytic deprotection produced the anomeric phosphate **4**, which was treated with diphenyl citronellol pyrophosphate.[10,11] The pyrophosphate exchange reaction took place readily in the presence of the unprotected sugar hydroxyls. Finally, the side chain protecting groups on the peptide were removed with TBAF, which also hydrolyzed the C-terminal methyl ester to give the desired product **5a**. It should be noted that **5a** is both acid- and base-sensitive. The synthesis minimizes exposure to acid and base, while providing for a convergent approach that allows independent modification of all three building blocks: the peptide, the carbohydrate, and the lipid. Thus,

(1) (a) Fan, C.; Moews, P. C.; Walsh, C. T.; Knox, J. R. *Science* **1994**, *266*, 439. (b) Benson, T. E.; Filman, D. J.; Walsh, C. T.; Hogle, J. M. *Nat. Struct. Biol.* **1995**, *2*, 644. (c) Jin, H. Y.; Emanuele, J. J.; Fairman, R.; Robertson, J. G.; Hail, M. E.; Ho, H. T.; Falk, P.; Villafranca, J. J. *Biochemistry* **1996**, *35*, 1423. (d) Skarzynski, T.; Mistry, A.; Wonacott, A.; Hutchinson, S. E.; Kelly, V. A.; Duncan, K. *Structure* **1996**, *4*, 1465. (e) Schonbrunn, E.; Sack, S.; Eschenburg, S.; Perrakis, A.; Krekel, F.; Amrhein, N.; Mandelkow, E. *Structure* **1996**, *4*, 1065. (f) Benson, T. E.; Walsh, C. T.; Hogle, J. M. *Biochemistry* **1997**, *36*, 806.

(2) (a) Gittins, J. R.; Phoenix, D. A.; Pratt, J. M. *FEMS Microbiol. Rev.* **1994**, *13*, 1. (b) Bupp, K.; van Heijenoort, J. *J. Bacteriol.* **1993**, *175*, 1841.

(3) (a) Pless, D. D.; Neuhaus, F. C. *J. Biol. Chem.* **1973**, *248*, 1568. (b) van Heijenoort, Y.; Gomez, M.; Derrien, M.; Ayala, J.; van Heijenoort, J. *J. Bacteriol.* **1992**, *174*, 3549.

(4) For a fluorescent assay to monitor MraY activity, see: Brandish, P. E.; Burnham, M. K.; Lonsdale, J. T.; Southgate, R.; Inukai, M.; Bugg, T. D. H. *J. Biol. Chem.* **1996**, *271*, 7609.

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(6) (a) Anderson, J. S.; Matsuhashi, M.; Haskin, M. A.; Strominger, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, *53*, 881. (b) Anderson, J. S.; Matsuhashi, M.; Haskin, M. A.; Strominger, J. L. *J. Biol. Chem.* **1967**, *242*, 3180. (c) Taku, A.; Fan, D. P. *J. Biol. Chem.* **1976**, *251*, 6154. (d) Mengin-Lecreulx, D.; Texier, L.; van Heijenoort, J. *Nucleic Acid Res.* **1990**, *18*, 2810. (e) Ikeda, M.; Wachi, M.; Jung, H. K.; Ishino, F.; Matsuhashi, M. *Nucleic Acid Res.* **1990**, *18*, 4014. (f) Mengin-Lecreulx, D.; Texier, L.; Rousseau, M.; van Heijenoort, J. *J. Bacteriol.* **1991**, *173*, 4625. (g) Miyao, A.; Yoshimura, A.; Sato, T.; Yamamoto, T.; Theeragool, T.; Kobayashi, Y. *Gene* **1992**, *118*, 147. (h) Ikeda, M.; Wachi, M.; Matsuhashi, M. *J. Gen. Appl. Microbiol.* **1992**, *38*, 53.(7) Although lipid I can be isolated with difficulty,[6b] MurG activity is typically assessed using crude membrane preparations in which the lipid I substrate is generated in situ, and then the incorporation of radiolabel from radiolabeled UDP-GlcNAc into lipid-linked products is monitored.[2b,6h] This type of assay is adequate for evaluating inhibitors but is not suitable for detailed mechanistic or structural studies.

(8) Chen, J.; Dorman, G.; Prestwich, G. *J. Org. Chem.* **1996**, *61*, 393.

(9) The protected pentapeptide was synthesized on a D-Ala-FMOC Sasrin resin (Bachem Biosciences) in 11 steps in an overall yield of 15%. Experimental details are provided in Supporting Information.

(10) Diphenyl citronellol pyrophosphate was generated in situ by treating citronellol phosphate with diphenyl chlorophosphate (Supporting Information). See also: Warren, C. D.; Jeanloz, R. W. *Methods Enzymol.* **1978**, *50*, 122.

(11) For other methods to form glycosyl pyrophosphates, see: (a) Imperiali, B.; Zimmerman, J. W. *Tetrahedron Lett.* **1990**, *31*, 6485. (b) Wittmann, V.; Wong, C.-H. *J. Org. Chem.* **1997**, *62*, 2144.

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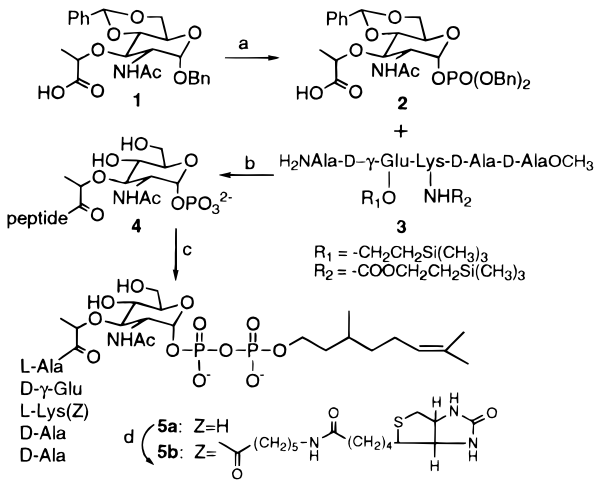
Scheme 2[a]



[a] (a) (1) 2 equiv of CCl_3CH_2OH, 1.5 equiv of DCC, 0.1 equiv of DMAP, THF, room temperature, 4 h, 80%; (2) H_2/Pd, EtOAc, room temperature, 0.5 h; 10 equiv of $PhCH(OCH_3)_2$, 0.1 equiv of TsOH, DMF, room temperature, 10 h, 81%, 2 steps; (3) 2 equiv of $^iPr_2NP(OBn)_2$, 4 equiv of ^{1}H-tetrazole, CH_2Cl_2, −20 °C → 0 °C, 0.5 h, then 5 equiv of mCPBA, −40 °C → 25 °C, 2 h, 75%; (4) Zn dust, 90% AcOH/H_2O, room temperature, 1 h, 91%. (b) (1) 2 equiv of HOBt, 2 equiv of Py-Bop, DIEA, DMF, 0 °C, 0.5 h, 92%; (2) H_2/Pd, CH_3OH, room temperature, 0.5 h, then DIEA. (c) (1) 2 equiv of (*R*)-(+)-*β*-citronellol-$OPO_3PO(OPh)_2$, py, DMF, room temperature, 48 h, 30%, 2 steps; (2) 20 equiv of TBAF, DMF, room temperature 24 h, 57%. (d) 2 equiv of 6-[(biotinoyl)amino]hexanoic acid succinimide ester, 5 equiv of $NaHCO_3$, H_2O/Dioxane, room temperature 2 h, 76%.

using the same general scheme we should be able to make a variety of compounds to define the requirements for substrate binding.

Initial attempts to use substrate **5a** in MurG activity assay revealed problems in separating radiolabeled product from excess labeled UDP-GlcNAc. The evidence suggests, however, that MurG is relatively insensitive to the identity of the third amino acid in the peptide chain.[12,13] Therefore, we attached biotin (Scheme 2)[14] to the ϵ amino group of the lysine residue so that radiolabeled product can be readily separated from other radioactive components in the reaction mixture using an avidin-derivatized resin (Tetralin Tetrameric Avidin Resin, Promega). The ability of MurG to recognize the biotin-labeled substrate **5b** was evaluated by counting the radioactivity that binds to the resin after incubation of various crude membrane preparations with **5b** and [^{14}C]UDP-GlcNAc.[15] Reaction is rapid and efficient with a bacterial culture that overexpresses MurG but barely detectable with a culture expressing only endogenous levels of MurG (Figure 1; cf. curves A and E).[16] Heat treating the overexpressing cell lysate prior to adding it to the substrates prevents the reaction (Figure 1; cf. A and D). Hence, the reaction depends on the presence of active MurG. Furthermore, both the initial reaction rate and conversion to product increase with the concentration of **5b** (Figure 1; cf. A, B, and C).

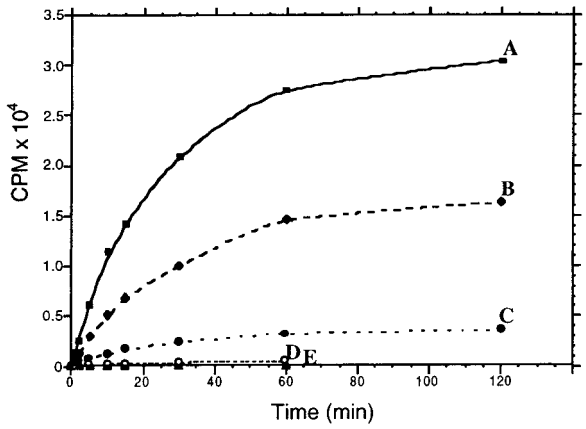


Figure 1. GlcNAc transfer as a function of [**5b**] and [active MurG]. All reactions were run in 100 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, with 0.5−1.0 μg total protein and 9.4 μM [^{14}C]UDP-GlcNAc (265 mCi/mmol). Reactions for curves A, B, C, and D were carried out using a cell lysate from a transformed BL21(DE3)pLysS strain that overexpresses MurG: (A, ■) 7.1 μM **5b**; (B, ◆) 3.5 μM **5b**; (C, ●) 0.71 μM **5b**; (D, ○) 7.1 μM **5b** + heat-treated cell lysate (65 °C, 5 min). Reactions for curve E were carried out using a BL21(DE3)pLysS cell lysate expressing only endogenous levels of MurG: (E, △) 7.1 μM **5b**.

In conclusion, the synthetic substrate functions efficiently in a direct assay for MurG activity despite having a different, and dramatically shorter, lipid chain. It should be possible to use this synthetic substrate to evaluate enzyme activity in overexpressing cell lysates following structural modifications to the *murG* gene that produce amino acid truncations, additions, deletions, or mutations. The synthetic substrate can also be used to assay for enzyme activity during purification, as well as for detailed mechanistic studies on wholly or partially purified enzyme. These types of experiments will lay the foundation for high-resolution structural analysis of MurG. In addition, by evaluating the ability of other synthetic substrates to compete with **5b** for [^{14}C]UDP-GlcNAc, it should be possible to identify simpler acceptors for use in direct screens for MurG inhibition.

Acknowledgment. This work was supported by Interneuron Pharmaceuticals. We thank Transcell Technologies for generously providing a plasmid containing *murG*.

Supporting Information Available: Experimental procedures and spectral data for the synthesis of the protected pentapeptide and diphenyl citronellol pyrophosphate; spectral data for compounds **4**, **5a**, and **5b** (18 pages). See any current masthead page for ordering information and Web access instructions.

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(12) *E. coli* strains (e.g., BL21) make a muramyl pentapeptide substrate with *meso*-diaminopimelic acid rather than L-lysine, but *E. coli* MurG accepts the lysine analogues.[6h,13] Fluorescently labeled analogs are also accepted by some strains: Weppner, W. A.; Neuhaus, F. C. *J. Biol. Chem.* **1978**, *253*, 472.

(13) White, D. *Physiology and Biochemistry of Prokaryotes*; Oxford Univ. Press: New York, 1995; pp 212-223.

(14) 6-[(biotinoyl)amino]hexanoic acid succinimide ester was purchased from Molecular Probes, Inc.

(15) Baker, C. A.; Poorman, R. A.; Kezdy, F. J.; Staples, D. J.; Smith, C. W.; Elhammer, A. P. *Anal. Biochem.* **1996**, *239*, 20.

(16) The *murG* gene was subcloned from a pET15b construct provided by Transcell Technologies, Inc. into a pET3a plasmid. MurG was overexpressed in the IPTG-inducible BL21(DE3)pLysS strain (Novagen). See: Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. *Methods Enzymol.* **1990**, *185*, 60.