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Chemoenzymatic synthesis of Park's nucleotide: toward the development of high-throughput screening for MraY inhibitors

Michio Kurosu,* Sebabrata Mahapatra, Prabagaran Narayanasamy and Dean C. Crick

Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 1682 Campus Delivery, Fort Collins, CO 80523-1682, USA

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Abstract—An efficient chemoenzymatic synthesis of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (Park's nucleotide) is reported. UDP-MurNAc is efficiently synthesized by a minimum number of protecting strategies. One-pot amino acid ligation reactions catalyzed by MurC, D, E, and F enzymes are amenable to scale-up production.

Although the machinery for cell wall peptidoglycan (PG) biosynthesis is considered a viable drug target, which step of PG biosynthesis represents the most promising drug target has not been defined.[1] In the past 40 years efforts to discover antibacterials which inhibit PG synthesis, including the cytosolic proteins (MurA–F), membrane bound proteins (MraY and MurG) and the transglycosylase/transpeptidases (penicillin-binding proteins or PBPs), which act extracellularly, have yielded a variety of chemical structures, most of which are natural products.[2] The most commonly exploited target in PG synthesis is the PBPs, which are inhibited by the β-lactams and glycopeptides. Unfortunately, these compounds are of little use in *Mycobacterium tuberculosis* infections due to the intrinsic β-lactamase activity and the impermeability of the mycolic acid layer of the cell wall in mycobacteria.[3] Other biosynthetic steps in PG synthesis are targeted by antibiotics including bacitracin, D-cycloserine and fosfomycin. These molecules are of limited usefulness in treating tuberculosis. Bacitracin, which inhibits prenyl diphosphate recycling after transglycosylation, has serious nephrotoxicity and is only used topically in human treatment. Fosfomycin, a MurA inhibitor, is ineffective against *M. tuberculosis* due to an alteration in a single amino acid residue in mycobacterial MurA and perhaps due to lack of uptake of this phosphonate. D-Cycloserine is used as an antituberculosis agent, inhibiting L-alanine racemase and dipeptidyl synthetase, preventing incorporation of D-alanyl-D-alanine into the pentapeptide side chains of Park's nucleotide.[4] However, D-cycloserine also generates central nervous system toxicity, limiting its usefulness.[5] Thus, PG synthesis appears to be a source of underexploited drug targets in mycobacterial pathogens.

In connection with our ongoing efforts to identify and validate new drug targets in MDR-tuberculosis, we are attempting to exploit PG biosynthesis. Bacterial cell wall PG biosynthesis consists of the following distinct steps. The synthesis of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (Park's nucleotide)[6] takes place in the cytosol in which MurA and MurB convert UDP-GlcNAc to UDP-MurNAc using pyruvate and NADPH as a reducing agent and other enzymes (MurC, D, E, and F) are ATP-dependent amino acid ligases that form the peptide bonds sequentially using L-alanine (L-Ala), D-glutamic acid (D-Glu), *meso*-diaminopimelic acid (*meso*-DAP), and D-alanyl-D-alanine (D-Ala-D-Ala), respectively. MraY and MurG are membrane-associated proteins that catalyze the decaprenyl (C_{50}) phosphorylation[7] of Park's nucleotide to form decaprenylpyrophosphoryl-MurNAc-L-Ala-γ-D-glu-*meso*-DAP-D-Ala-D-Ala (lipid I) and the transfer of *N*-acetylglucosamine (GlcNAc) to the C4-hydroxyl group of Lipid I to form Lipid II, respectively. Lipid II is the final monomeric building block in bacterial cell wall biosynthesis. In mycobacteria lipid II is composed of a complex mixture of modified muramyl and peptide moieties linked to decaprenyl phosphate and it appears that most of these modifications take place on lipid II

* Corresponding author. Tel.: +970 491 7628; fax: +970 491 1815; e-mail: michio.kurosu@colostate.edu

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rather than a precursor such as Park's nucleotide.[8] Lipid II is then transported to the extracellular face of the plasma membrane by a currently undefined mechanism. This externalized lipid II is polymerized and cross-linked by the transglycosylase and transpeptidase activities of the PBPs.

Many inhibitors of cytoplasmic MurA–F have been reported; however, few have been developed to drug-like lead molecules.[9] Although there is no antibiotic or a small molecule directed against MraY being pursued for drug development, inhibitors of this enzyme such as tunicamycin, caprazamycin, mureidomycin, liposidomycin, FR900493, and their congeners exhibited significant antibacterial activity in vitro.[10] Encouraged by these excellent antibacterial activity profiles, we have developed an efficient synthesis of the common structure of these antibiotics and commenced the generation of relatively large-membered compound library based on the structure of FR900493 (Fig. 1).[11]

In order to assay the generated library molecules, a high-throughput screening (HTS) assay for MraY is required. Thus far, several screening methods for MraY inhibitors have been developed including: (1) monitoring the transfer of phosphoryl-MurNAc-pentapeptide from Park's nucleotide to undecaprenyl phosphate; this assay requires fluorescent or radiolabeled Park's nucleotide,[12] (2) measuring the exchange reaction between [^{3}H]UMP to Park's nucleotide, which occurs as result of the reversibility of the MraY catalyzed reaction; this assay involves separation of [^{3}H]uridine after the treatment of alkaline phosphatase,[13] (3) an indirect assay using a coupled MraY–MurG in which biotinylated Park's nucleotide and [^{14}C]UDP-GlcNAc were used as substrates; in this assay, biotinylated lipid II was separated by using streptavidin-coated beads,[14] (4) an assay using HP20ss hydrophobic beads for isolating the generated radiolabeled lipid I,[15] (5) a microplate-based assay for using a radiolabeled Park's nucleotide,[16] and (6) a scintillation proximity assay using wheat germ agglutin-coated beads to capture the lipid I from radiolabeled Park's nucleotide.[17] Thus, most reported MraY assays require radiolabeled Park's nucleotide. The requirement for use of a radiolabel is a limitation of applicability of these assays in a HTS because the synthesis of radiolabeled-Park's nucleotide in whole cells through the incorporation of radiolabeled-amino acids: (1) involves time-consuming separations of the product, and (2) resulted in very low yield. In addition, handling and disposal of radioactive waste is expensive and time consuming. Therefore, we are developing coupled assays for PG synthesis inhibitors which are not dependent on radioactive substrates, but which are coupled to the formation of highly UV absorbent of fluorescent compounds for HTS.

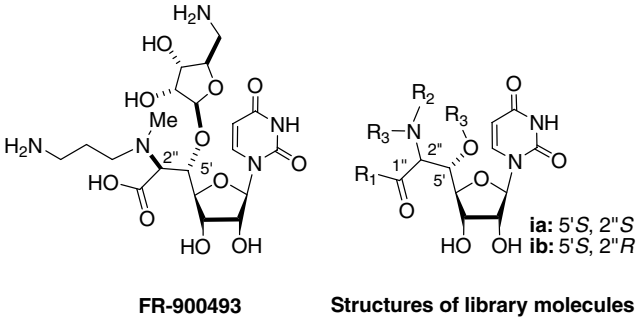


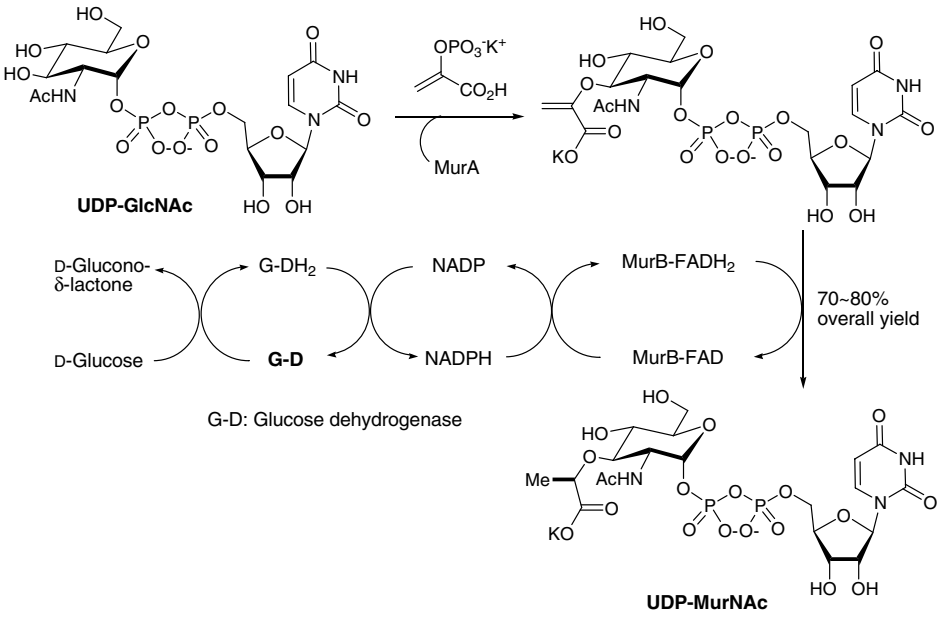
Figure 1. The structures of FR-90093 and uridine-amino alcohol library.

A requirement for HTS is a significant supply of substrate. For example, in order to supply gram-quantity of Park's nucleotide for establishing an assay for a HTS against MraY, an efficient method for the synthesis of Park's nucleotide is indispensable.[18] We previously synthesized up to 100 mg of Park's nucleotide enzymatically; however, the cost of starting materials (UDP-GlcNAc, NADPH) for a large scale synthesis using this method is prohibitive.[19] A total synthesis of Parks nucleotide has also been reported, but involves a long sequence and resulted in relatively low yields.[18] In order to avoid these practical issues we developed a scalable chemoenzymatic synthesis of Park's nucleotide.

We first examined the enzymatic synthesis of UDP-MurNAc from UDP-GlcNAc according to Jin et al.[20] using recombinant MurA and MurB cloned from *Escherichia coli*. It was, however, realized that 5 mol % of MurB and 200 mol % of NADPH are necessary to achieve >80% conversion. In order to avoid using stoichiometric amounts of expensive external reducing agent, NADPH, we investigated a regeneration system in which a glucose dehydrogenase was utilized to regenerate NADPH from NADP. As a result of extensive reaction optimization, it was concluded that ~10 mol % of NADPH is necessary in efficient regeneration of $FADH_2$ (co-factor of MurB) with 10–20 mol % of glucose dehydrogenase[21] in the presence of D-glucose; under these conditions UDP-GlcNAc was converted to UDP-MurNAc in 70–80% yield after an anion exchange chromatography (Scheme 1). Although the methodology could be used to synthesize significant amounts of UDP-MurNAc, application of this catalytic process to a larger scale synthesis may not be practical. In addition, the expense of the required UDP-GlcNAc is also a consideration when using an enzymatic synthesis of UDP-MurNAc, as noted above.

Thus, we turned our attention to a more practical chemical synthesis of UDP-MurNAc from GlcNAc. However, no chemical synthesis of UDP-MurNAc has been reported.[22] In an efficient chemical synthesis of UDP-MurNAc, the following issues were considered: (1) limited use of protecting group chemistry, and (2) a minimum number of chromatographic purifications in the synthetic sequence. Although phosphorylation reactions of monophosphates with commercially available uridine 5′-morpholinophosphonate have been reported, they are extremely slow and resulted in poor conversion.[23] Therefore, an alternative UDP-5′-monophosphate derivative was required to improve the reaction rate and the conversion yield.

As illustrated in Scheme 2, our synthesis began with the S_N2 addition of (*S*)-methyl-2-chloropropanonate with

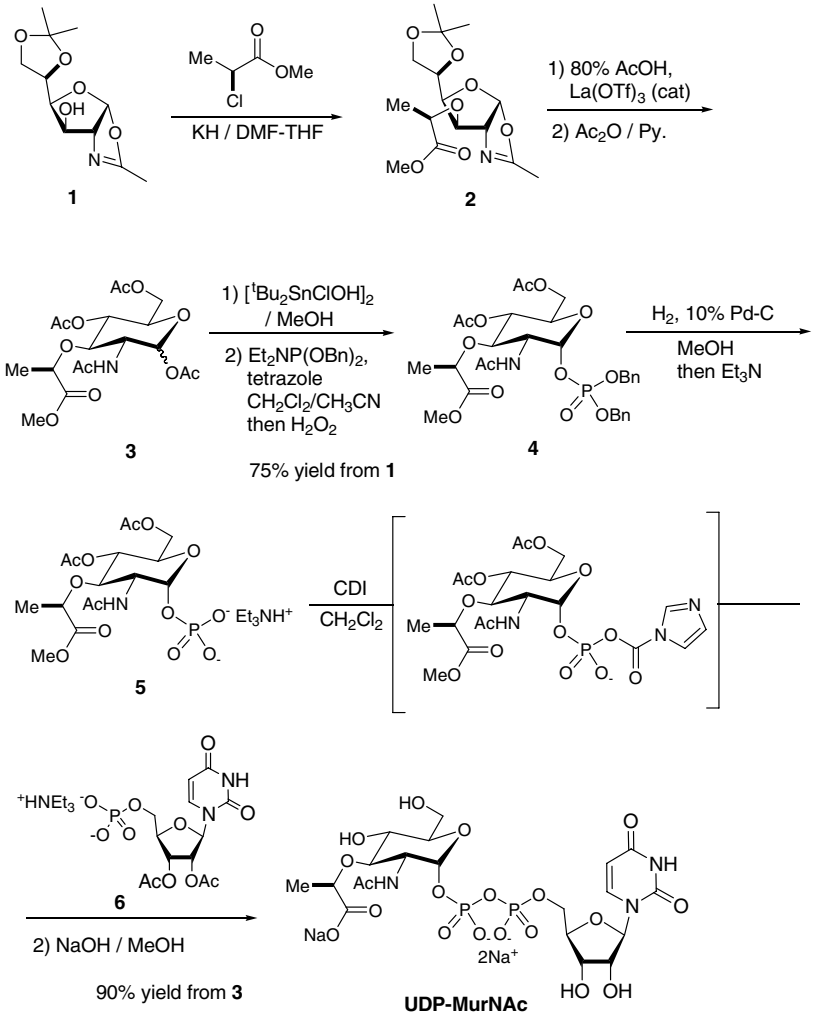


Scheme 1. One-pot enzymatic synthesis of UDP-MurNAc.

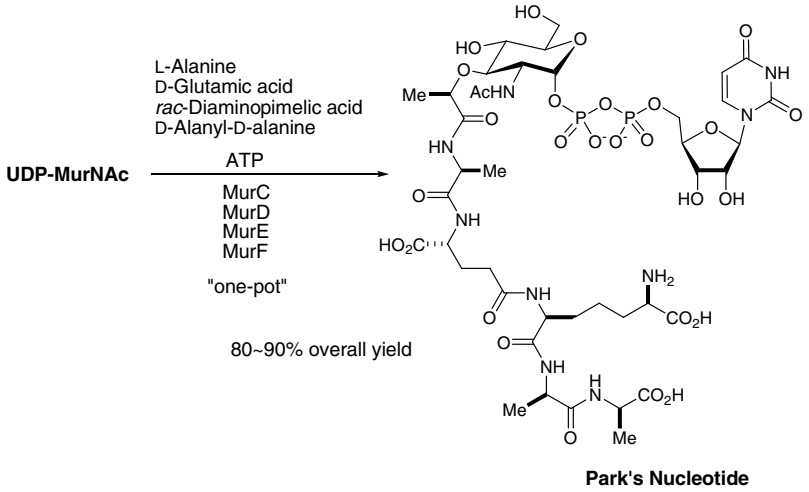
the oxazoline **1**, which was readily synthesized by a kinetically controlled acetonide formation reaction of GlcNAc[24], to provide the desired five-membered muramic acid derivative **2** in over 90% yield. This was utilized in the next reaction without purification. **2** was converted to the corresponding pyranoside with 80% AcOH in the presence of 3 mol % of $La(OTf)_3$. Acetylation of the free-hydroxyl groups followed by selective deacetylation via 3 mol % of $[^tBu_2SnClOH]_2$[25] in MeOH underwent smoothly to provide the free-alcohol at anomeric position. α-Selective phosphite formation was achieved by using diethyl-*N*,*N*-diethylphosphoramidite in the presence of tetrazole in a mixture of CH_2Cl_2 and acetonitrile (3/1) at −15 °C, followed by oxidation of the generated phosphite with H_2O_2[26] gave the corresponding phosphate **4** in 75% overall yield from **1**. Significantly, the phosphoryl-MurNAc derivative **4** was synthesized by a single chromatographic purification for a five-steps sequence from **1**. Debenzylation of **4** followed by the addition of triethylamine yielded the corresponding triethylammonium phosphate **5** in quantitative yield which was then subjected to the carbonyldiimidazole promoted diphosphate-formation reaction[27] with **6**[28] to provide the fully-protected UDP-MurNAc within 0.5 h in nearly quantitative yield. Global deprotection with aq NaOH and purification by a sephadex chromatography afforded UDP-MurNAc in 90% overall yield from **4**. The chemically synthesized UDP-MurNAc was determined by ^1H NMR, reverse-phase TLC ($MeOH:CHCl_3:H_2O:Et_3N = 3:2:1:0.05$), and MS which was identical to a sample that was obtained by the enzymatic pathway (Scheme 1).

As illustrated in Scheme 3, Park's nucleotide was efficiently synthesized enzymatically by sequentially adding L-Ala, D-Glu, *rac*-DAP, and D-Ala-D-Ala to UDP-MurNAc using purified recombinant MurC, MurD, MurE, and MurF in a single reaction mixture. The *E. coli* Mur enzymes (MurC, MurD, MurE, and MurF) used for the synthesis were overexpressed and purified using the Impact CN system.[29] The reaction mixture containing 270 μmol of UDP-MurNAc, 1 mM of L-Ala, D-Glu, *rac*-DAP, and D-Ala-D-Ala, 50 mM TAPS (pH 8.0), 5 mM $MgCl_2$, 2.5 mM ATP and MurC, MurD, MurE, and MurF (75 μg/ml each) in TAP buffer (30 mL) was incubated at 30 °C for 12 h, deproteinated by ultrafiltration and the filtrate was loaded on a Q-Sepharose column equilibrated with 20 mM ammonium acetate buffer (pH 9). The bound material was eluted with a 20–1000 mM gradient of ammonium acetate (pH 9). The fractions were monitored for UDP content at A_{262} and the UDP containing fractions were analyzed by TLC (using UV absorption). Park's nucleotide containing fractions were lyophilized to remove ammonium acetate to provide 216–243 μmol of Park's nucleotide whose purity was established by a reverse phase HPLC as well as ^1H NMR analyzes.[30] In this enzymatic synthesis, *rac*-DAP was used and MurE exclusively utilized the (*S*,*S*)-configuration diastereomer. We also demonstrated that the various intermediates such as UDP-MurNAc-L-Ala, UDP-MurNAc-L-Ala-γ-D-Glu, UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP can also be synthesized simply by leaving appropriate enzymes and reagents out of the reaction. UDP-MurNAc-L-[^{14}C]Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala was also synthesized using a similar but scaled down reaction in which L-Ala was replaced with L-[^{14}C]Ala in over 80% yield.

In conclusion, the chemoenzymatic synthesis of Park's nucleotide demonstrated here: (1) includes an efficient chemical synthesis of UDP-MurNAc and one-pot amino acid ligation reactions by using recombinant MurC, D, E, F enzymes, (2) is a flexible and high-yielding process. For example, instead of bulk synthesis, radioactive compounds can reliably be synthesized by replacing **6** in Scheme 2 and amino acids in Scheme 3 with the corre-



Scheme 2. Chemical synthesis of UDP-MurNAc.



Scheme 3. Enzymatic synthesis of Park's nucleotide from UDP-MurNAc.

sponding radioactive molecules. Radioactive Park's nucleotides are useful biochemical tools for studying PG biosynthesis, kinetic characterization of MraY or use in low- to medium-throughput screening assays. In addition, substrates for study of MurC–F or corresponding HTS can be prepared by simply leaving appropriate enzymes and reagents out of the reaction as described above.

Acknowledgments

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