Biosynthesis of a water-soluble lipid I analogue and a convenient assay for translocase I

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Abstract

Translocase I (MraY/MurX) is an essential enzyme in growth of the vast majority of bacteria that catalyzes the transformation from UDP-MurNAc-pentapeptide (Park’s nucleotide) to prenyl-MurNAc-pentapeptide (lipid I), the ﬁrst membrane-anchored peptidoglycan precursor. MurX has received considerable attention in the development of new tuberculosis (TB) drugs due to the fact that the MurX inhibitors kill exponentially growing Mycobacterium tuberculosis (Mtb) much faster than clinically used TB drugs. Lipid I isolated from Mtb contains the C50-prenyl unit that shows very poor water solubility; thus, this chemical characteristic of lipid I renders MurX enzyme assays impractical for screening and lacks reproducibility of the enzyme assays. We have established a scalable chemical synthesis of Park’s nucleotide-Ne-dansylthiou-rea 2 that can be used as a MurX enzymatic substrate to form lipid I analogues. In our investigation of the minimum structure requirement of the prenyl phosphate in the MraY/MurX-catalyzed lipid I analogue syn- thesis with 2, we found that neryl phosphate (C10 phosphate) can be recognized by MraY/MurX to generate the water-soluble lipid I analogue in quantitative yield under the optimized conditions. Here, we report a rapid and robust analytical method for quantifying MraY/MurX inhibitory activity of library molecules.

**Introduction**

The eradication of tuberculosis (TB)2 remains a prominent chal- lenge for basic, translational, and clinical research scientists [1]. Once thought to be under control, tuberculosis case reports are increasing worldwide and the disease poses a major global public health threat. In 2011, 8.7 million people were infected with *Mycobacterium tuberculosis* (Mtb) and 1.4 million people died from TB [2,3]. One-third of the 42 million people living with HIV/AIDS world- wide are coinfected with Mtb [4,5]. Clinical responses of multidrug- resistant (MDR)–TB patients to the ﬁrst-line drugs have been poor, and in some cases there is no response at all. The World Health Organization (WHO) estimated that 650,000 new cases of MDR–TB emerge each year, and 27 countries around the world account for dextrose, and catalase; ADC, albumin, dextrose, and catalase; DMSO, dimethyl sulfoxide.

86% of the MDR–TB burden. An outbreak of extensively drug-resis- tant (XDR)–Mtb was reported in 2006 [3,6]. For MDR strains of Mtb, treatment length of TB chemotherapy can be at least 20 to

28 months. The treatment of XDR–TB takes substantially longer than that of MDR–TB [4,7]. Thus, it is signiﬁcantly important to discover promising approaches to shorten the current TB drug regimen. In in vitro time kill assessment experiments, U.S. Food and Drug Administration (FDA)-approved TB drugs required 11 to 14 days to kill exponentially growing Mtb at 2–4 x MIC (minimum inhibitory concentration) values. On the other hand, several translocase I (MraY/MurX, hereafter referred to as MurX for Mtb translocase I) inhibitors have been known to kill more than 95% of Mtb in 2 to 5 days at MIC or 2–4 x MIC values [8,9]. Because peptidoglycan (PG) is an essential bacterial cell wall polymer, the machinery for PG biosynthesis provides a unique and selective target for antibiotic action. The biosynthesis of PG of Escherichia coli has been discussed extensively in reviews by van Heijenoort [10–12]. Most of the genes involved in peptidoglycan biosynthesis in E. coli are known, and orthologues have been identiﬁed in the gram-positive genomes. However, very few genes responsible for the unique features of mycobacterial PG to diversify the cell wall structure have been known. Detailed analyses of the components of mycobacterial PG revealed that it contains a variety of modiﬁed molecules, including (i) an N-glycolyl (NGlyc) group in addition to an N-acetyl (NAc) group on the muramic acid (Mur), (ii) amidation of the carboxylic acids in the peptide moieties of PG, and (iii) additional glycine or ser- ine residues [13–15]. Interestingly, the N-glycolylated muramic acid predominates in mycobacteria [13] (Fig. 1). To date, only a few enzymes in PG biosynthesis, such as the transpeptidase of penicillin binding proteins (PBPs), have been studied extensively. Thus, the machinery for PG synthesis is still considered to be a source of unex- ploited drug targets. However, most of drugs associated with cell wall biosynthesis might not reduce treatment time of a TB drug reg- imen because the dormant or nonreplicating Mtb is not actively syn- thesizing cell walls [16]. On the contrary, a fast bactericidal effect of MurX inhibitors is very attractive to develop new TB drugs that reduce the time frame for effective anti-TB chemotherapy [8]. MurX catalyzes the transformation of UDP-MurNGlyc-pentapeptide and UDP-MurNAc-pentapeptide (Park’s nucleotide) to the corresponding lipid I using decaprenyl (C50) phosphate in *Mycobacterium* spp. [17,18]. This process is believed to be a reversible process in which E. coli MraY catalyzes an exchange reaction between UMP and lipid I to form Park’s nucleotide in vitro [19].

Isolation and quantitation of Park’s nucleotide and lipid I from in vitro MraY/MurX assay reaction mixtures are time-consuming processes [17]. In addition, preparation of Mtb Park’s nucleotide via semi-puriﬁed Mur enzymes is not amenable to multigram scale-up, and the acquisition cost of enough decaprenyl phosphate for medium- to high-throughput screenings is very high. To date, several screening methods for MraY/MurX inhibitors have been reported, including (i) monitoring the transfer of phosphoryl-Mur- NAc-pentapeptide using ﬂuorescent or radiolabeled Park’s nucleo- tide and/or undecaprenyl phosphate [19], (ii) measuring the exchange reaction between [3H]UMP and Park’s nucleotide that requires separation of [3H]uridine after the treatment of alkaline phosphatase [20,21], (iii) an indirect assay using a coupled MraY/ MurG that requires biotinylated Park’s nucleotide and [14C]UDP- GlcNAc [22], (iv) an assay using HP20ss hydrophobic beads for iso- lating the generated radiolabeled lipid I [23], (v) a microplate-based assay using a radiolabeled Park’s nucleotide [24], and (vi) a scintilla- tion proximity assay using wheat germ agglutinin-coated beads to capture the lipid I from a radiolabeled Park’s nucleotide [25]. Although several assay methods were reported to be amenable to a high-throughput screening (HTS) assay for MraY [19,25,26], in our hands extraction of water-insoluble lipid I derivative from assay media is essential. In our attempt at developing reliable in vitro MraY/MurX assay, we concluded that the reported assays need fur- ther optimization to be robust statistical methods that can identify MraY/MurX inhibitors routinely with IC50 values. We established an efﬁcient synthetic method for the generation of a sufﬁcient amount of ﬂuorescent Park’s nucleotide probes for HTS [27,28], and tested the Park’s nucleotide probes in MurX-catalyzed lipid I ana- logue synthesis with decaprenyl and truncated prenyl phosphates. Surprisingly, under the optimized conditions, the water-soluble lipid I-neryl (C10) analogue could be biosynthesized efﬁciently with the Park’s nucleotide probes and neryl phosphate. In the current work, we report a convenient and reliable enzyme assay for MurX to identify antimycobacterial MurX inhibitor molecules.

**Materials and methods**

Chemicals

Difco Middlebrook 7H10 agar, Middlebrook 7H9 broth, tryptic soy agar, tryptic soy broth, Mops, tris(hydroxymethyl)aminometh- ane, 2-mercaptoethanol, sucrose, and Triton X-100 were purchased from Sigma–Aldrich. ADC enrichment was purchased from Fisher Scientiﬁc. Magnesium chloride and potassium chloride were obtained from VWR. All reagents and solvents were commercial grade and were used as received without further puriﬁcation unless otherwise noted. Flash chromatography was performed with What- man silica gel (Purasil 60 Å, 230–400 mesh). Analytical thin-layer chromatography was performed with 0.25-mm coated commercial silica gel plates (EMD, Silica Gel 60F254) visualizing at 254 nm or developed with ceric ammonium molybdate or anisaldehyde solu- tions by heating on a hot plate. 1H NMR spectral data were obtained using 400- and 500-MHz instruments. 13C NMR spectral data were obtained using 100- and 125-MHz instruments. For all NMR spectra, d values are given in ppm and J values are given in Hz.

MurX/MraY assay substrates

Park’s nucleotide-Ne-dansylthiourea 2, neryl-lipid I-Ne-dansylthiourea 5, and neryl phosphate (6) were chemically synthesized from the corresponding starting materials.

Neryl phosphate (6)

To a solution of phosphoric acid (98 mg, 1.0 mmol), pyridine (0.40 ml, 5.0 mmol), and nerol (1.8 ml, 10 mmol) was added trieth- ylamine (0.28 ml, 2.0 mmol). After being stirred for 30 min, acetic anhydride (0.19 ml, 2.0 mmol) was added to the reaction mixture. The reaction mixture was stirred at 80 oC for 12 h, and the reaction was cooled to room temperature. The reaction was quenched with water (5 ml) and stirred for 1 h at 80 oC. The reaction mixture was cooled to room temperature, and the aqueous phase was extracted with ether (5 ml x 3). Lyophilization of the aqueous phase gave the crude product. Puriﬁcation by DOWEX 50WX8 afforded neryl phosphate (6)-mono ammonium salt (0.17 g, 74%) as a white solid [29]. This reaction could readily be scaled up to multigrams of neryl phosphate-mono ammonium salt.

1H NMR (400 MHz, D2O2) d 5.33 (td, J = 7.3, 1.6 Hz, 1H), 5.14–5.04 (m, 1H), 4.27 (t, J = 7.5 Hz, 2H), 2.10–2.00 (m, 4H), 1.67 (d, J = 1.2 Hz, 3H), 1.59 (s,

3H), 1.53 (s, 3H); 13C NMR (101 MHz, D2O) d 142.75, 133.95,

123.84, 120.62, 120.54, 61.88, 61.83, 31.18, 25.92, 24.81, 22.57,

16.91; 31P NMR (162 MHz, D2O) d 3.72; LRMS (EI) calc’d for

C10H20O4P (M + H+): 235.11, found: 235.05.

Park’s nucleotide-Ne-dansylthiourea 2

Park’s nucleotide was synthesized according to the previously reported procedure [27]. To a stirred solution of N-acetyl Park’s nucleotide (3.0 mg, 2.6 lmol) in 0.1 M aqueous NaHCO3 solution (0.10 ml) was added 5-(dimethylamino)-N-(4-isothiocyanatophenyl)naphthalene-1-sulfonamide (3.0 mg, 7.8 lmol) in dimethyl-formamide (DMF, 0.05 ml). After being stirred for 2.5 h at room temperature, the reaction mixture was ﬁltered. The ﬁltrate was puriﬁed by reverse-phase high-performance liquid chromatography (HPLC column: HYPERSIL GOLD (175 A, 12 lm, 250 x 10 mm); solvents: a gradient elution of 0:100 to 30:70 CH3- CN/0.05 M aqueous NH4HCO3 over 30 min; ﬂow rate: 2.0 ml/min; ultraviolet (UV): 350 nm] to afford 2 (2.4 mg, 60%; retention time:

29 min). Similarly, N-acetyl Park’s nucleotide (500 mg) was con- verted to 2 in 75% yield. 1H NMR (400 MHz, D2O) d 8.43 (d, J = 8.6 Hz, 1H), 8.39 (d, J = 8.5 Hz, 1H), 8.23 (d, J = 7.3 Hz, 1H),

7.89 (d, J = 8.1 Hz, 1H), 7.67 (t, J = 8.1 Hz, 1H), 7.59 (t, J = 8.0 Hz,

1H), 7.37 (d, J = 7.6 Hz, 1H), 6.98–6.93 (m, 4H), 5.94 (d, J = 3.7 Hz,

1H), 5.89 (d, J = 8.1 Hz, 1H), 5.45 (dd, J = 7.2, 3.1 Hz, 1H), 5.28 (dd,

J = 7.2, 2.9 Hz, 1H), 4.33 (d, J = 3.3 Hz, 2H), 4.30 (d, J = 7.3 Hz, 1H),

4.27–4.22 (m, 2H), 4.22–4.16 (m, 3H), 4.16–4.08 (m, 3H), 4.07 (q, J = 7.2 Hz, 1H), 3.96–3.90 (m, 1H), 3.88–3.71 (m, 3H), 3.65–3.57 (m, 1H), 3.48–3.36 (m, 2H), 3.33 (s, 1H), 2.82 (s, 6H), 2.30–2.22 (m, 2H), 2.21 (s, 3H), 2.16–2.05 (m, 1H), 1.90–1.79 (m, 1H), 1.78–

1.63 (m, 2H), 1.53–1.42 (m, 2H), 1.37 (d, J = 7.1 Hz, 3H), 1.36 (d, J = 6.7 Hz, 3H), 1.31 (d, J = 7.3 Hz, 3H), 1.28 (d, J = 7.3 Hz, 3H); LRMS (EI) calc’d for C59H83N12O28P2S2 (M + H+): 1533.44, found: 1533.80.

Neryl-lipid I-Ne-dansylthiourea 5

Lipid I-neryl analogue was synthesized according to the reported procedure with a minor modiﬁcation [27,28]. Thiourea formation of lipid I-neryl analogue was performed under the same conditions for the synthesis of 2. The crude product was puriﬁed by reverse-phase HPLC column: HYPERSIL GOLD (175 A, 12 lm, 250 x 10 mm); solvent: a gradient elution of 20:80 to 50:50 CH3- CN/0.05 M aqueous NH4HCO3 over 30 min; ﬂow rate: 2.0 ml/min; UV: 350 nm] to afford 5 (2.0 mg, 70%; retention time: 20 min).

1H NMR (400 MHz, D2O) d 8.46 (d, J = 8.6 Hz, 1H), 8.35 (d, J = 8.8 Hz, 1H), 8.29 (d, J = 7.8 Hz, 1H), 7.73 (t, J = 8.1 Hz, 1H), 7.62 (t, J = 8.2 Hz, 1H), 7.42 (d, J = 7.3 Hz, 1H), 7.04–7.00 (m, 4H),

5.45–5.41 (m, 1H), 5.39–5.35 (m, 1H), 5.08–5.01 (m, 2H), 4.44–

4.38 (m, 2H), 4.30 (q, J = 6.9 Hz, 2H), 4.23 (q, J = 7.1 Hz, 2H), 4.18–

4.04 (m, 6H), 3.96–3.90 (m, 2H), 3.86 (dd, J = 12.0, 1.9 Hz, 1H), 3.81 (d, J = 4.4 Hz, 1H), 3.78 (d, J = 5.5 Hz, 1H), 3.74 (d, J = 9.3 Hz, 1H),

3.60 (t, J = 9.6 Hz, 1H), 3.49–3.40 (m, 2H), 2.85 (s, 6H), 2.29–2.23 (m, 2H), 2.05–1.97 (m, 2H), 1.94 (s, 3H), 1.89–1.80 (m, 2H), 1.74 (d, J = 28.0 Hz, 2H), 1.66 (s, 3H), 1.59 (s, 3H), 1.51 (s, 3H), 1.38 (d, J = 6.9 Hz, 3H), 1.36 (d, J = 6.7 Hz, 3H), 1.31 (d, J = 7.2 Hz, 3H),

1.28 (d, J = 7.2 Hz, 3H); LRMS (EI) calc’d for C60H89N10O23P2S2

(M + H+): 1443.50, found: 1443.90.

Bacterial strains and growth of bacteria

M. tuberculosis (H37Rv) was obtained through BEI Resources, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). Mycobacterium smegmatis (ATCC

607), Staphylococcus aureus (ATCC BAA-1556), and E. coli K-12 (ATCC 29425) were obtained from American Type Culture Collection (ATCC). A single colony of bacterial strain was obtained on a Difco Middlebrook 7H10 nutrient agar enriched with 10% oleic acid, albu- min, dextrose, and catalase (OADC for M. tuberculosis), with albumin, dextrose, and catalase (ADC for M. smegmatis), and on tryptic soy agar (for E. coli and S. aureus). Seed cultures were obtained in Middle- brook 7H9 broth enriched with OADC (for M. tuberculosis), with ADC (for M. smegmatis), and in tryptic soy broth (for E. coli and S. aureus). Each bacterium was grown to mid-log phase.

Preparation of membrane fraction P-60 containing MurX/MraY

M. tuberculosis cells were harvested by centrifugation (4700 rpm) at 4 oC followed by washing with 0.9% saline solution (thrice), and approx. 5 g of pellet (wet weight) was col- lected. The washed cell pellets were suspended in homogenization buffer (containing 50 mM Mops [pH 8.0], 0.25 M sucrose, 10 mM MgCl2, and 5 mM 2-mercaptoethanol) and disrupted by probe sonication on ice (10 cycles of 60 s on and 90 s off). The resulting sus- pension was centrifuged at 1000g for 10 min at 4 oC to remove unbroken cells. The supernatant was centrifuged at 25,000g for 40 min at 4 oC (3 or 4 times). All pellets in each tube were pooled, and a second sonication was performed (10 cycles of 60 s on and 90 s off). The lysate was centrifuged once at 25,000g for 1 h, and the supernatant was subjected to ultracentrifugation at 60,000g for 1 h at 4oC. The supernatant was discarded, and the membrane fraction containing MurX enzyme (P-60) was suspended in the Tris–HCl buffer (pH 7.5) containing 2-mercaptoethanol [30,31]. Total protein concentrations were approximately 8 to 10 mg/ml [32]. Aliquots were stored in Eppendorf tubes at -80 oC. Similarly, the membrane fractions containing MraY enzyme (P-60) were pre- pared from M. smegmatis, S. aureus, and E. coli, respectively.

MurX/MraY assay

Park’s nucleotide-Ne-dansylthiourea 2 (2 mM stock solution, 3.75 ll [75 lM]), MgCl2 (0.5 M, 10 ll [50mM]), KCl (2 M, 10 ll [200mM]), Triton X-100 (0.5%, 11.25 ll), Tris buffer (pH 8.0, 50 mM, 2.5 ll), neryl phosphate (6, 10 mM, 45 ll), and inhibitor (0–100 lM, in dimethyl sulfoxide [DMSO, 2.5 ll]) were place in a 500-ll Eppendorf tube. To a stirred reaction mixture, P-60 (15 ll) was added (total volume of reaction mixture: 100 ll). The reaction mixture was incubated for 1 h at room temperature (26 oC) and quenched with CHCl3 (200 ll). Two phases were mixed via vortex and centrifuged at 25,000g for 10 min. The upper aqueous phase was assayed via reverse-phase HPLC. The water phase (10 ll) was injected into HPLC (solvent: CH3CN/

0.05 M aq. NH4HCO3 = 25:75; UV: 350 nm; ﬂow rate: 0.5 ml/ min; column: Kinetex 5 lm C8, 100 Å, 150 x 4.60 mm), and the area of the peak for lipid I-neryl derivative 5 was quantiﬁed to

obtain the IC50 value. The IC50 values were calculated from plots of the percentage product inhibition versus the inhibitor concentration.

Kinetic parameter evaluation via MurX/MraY activity assay

Evaluation of kinetic parameters was performed through MurX- or *M. smegmatis* MraY-catalyzed lipid I synthesis. Km and Vmax were determined by Michaelis–Menten enzyme kinetics. The correlation (Michaelis–Menten plot) between the concentrations of Park’s nucleotide-dansylthiourea 2 (x axis) and rate (V) of lipid I formation (y axis) was obtained using GraphPad Prism software [33,34].

Compounds

All antimycobacterial molecules screened against MurX were synthesized in our laboratory except for tunicamycin (Sigma) and vancomycin (Sigma). All molecules were diluted with DMSO to be a concentration of 1 mg/100 ll (stock solution). The MurX assays developed here were tolerated to 2.5% of DMSO concentra- tions in total volume of the reaction solution (100 ll). The maxi- mum tolerated concentration for DMSO in MurX-catalyzed neryl lipid I-Ne-dansylthiourea 5 has not been determined

Determination of MIC values

*M. tuberculosis* was cultured to be an optical density of 0.4 to 0.5. Each compound (8 ll) stored in DMSO (1 mg/100 ll) was placed in a sterile 96-well plate, and a serial dilution was conducted with the culturing broth (total volume of 100 ll). The bacterial suspension (100 ll) was added to each well (total volume of 200 ll). The bacterial culture in a plate treated or not treated with compounds was incubated for 14 days at 37 oC in a shaking incubator (120 rpm). Resazurin (0.01%, 20 ll) was added to each well and incubated at 37 oC for 5 h. The MIC values were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) method (pink = growth, blue = no visible growth). The absorbance of each well was also measured at 570 and 600 nm via a microplate reader.

Results and discussion

A number of selective MraY inhibitors from natural sources have been reported [18,35–40]. The major source of future devel- opments resides in the nucleoside-based inhibitors, which are sub- divided into ﬁve classes. Tunicamycin is a relatively weak MurX inhibitor [26] and did not show strong antimycobacterial activity. Caprazamycin, muraymycins, liposidomycin, capuramycin, mure- idomycins, pacidamycins, and their congeners were reported to exhibit signiﬁcant MraY enzyme inhibitory activity in vitro [18]. Capuramycin showed activity speciﬁc to Mycobacterium spp. Anti- microbial spectrum focused against Mtb (selective antimycobacte- rial agent) is preferable for TB chemotherapy due to the fact that TB chemotherapy requires a long regimen, so that broad-spectrum anti-TB agents may cause resistance to other bacteria during TB chemotherapy. Most of the reported MraY inhibitors were compet- itive to Park’s nucleotide but not to prenyl phosphate. Because capuramycin is a well-characterized MraY/MurX inhibitor that showed activities focused against Mycobacterium spp. [39], we selected positive and negative controls from capuramycin deriva- tives to develop a convenient MurX assay method, and the new MurX assay developed here was planned to demonstrate by screening antimycobacterial uridyl-peptide library molecules.

N-Glycolylated muramic (MurNGlyc) acid predominates in M. tuberculosis, and MurNGlyc is the major muramyl building block in mycobacterial cell walls (Fig. 1) [13,40]. Thus, it is of importance to characterize speciﬁcity of MurX against N-glycolyl Park’s nucle- otide and N-acetyl Park’s nucleotide for discovery of novel antimy- cobacterial MurX inhibitors. To understand the tolerance of MurX in the structure of Park’s nucleotide, we have synthesized a series of Park’s nucleotide analogues according to our established chemo- enzymatic or total chemical syntheses [17,27,28]. We and other groups have demonstrated that the dansyl- or ﬂuorescein isothio- cyanate (FITC)-conjugated N-acetyl Park’s nucleotides can be applied to MraY enzyme assays with the MraY obtained from gram-negative organisms and some gram-positive organisms [40]. In our preliminary studies, the membrane fraction containing MraY or MurX enzyme (P-60) obtained from E. coli, S. aureus, M. smegmatis, or Mtb could transfer the ﬂuorescent Park’s nucleotide conjugates to the corresponding undecaprenyl lipid I analogues with undecaprenyl phosphate in 5 to 25% yields after 1 h of incubation [31]. These results were in accordance with the data previously reported by several other groups [19,20]. It was exper- imentally proved that MurX was also tolerated in the structure of the C6 position (Ne) of lysine moiety of Park’s nucleotides.

To further study tolerance of MurX against the structure of the N-acyl moiety of muramic acid in Park’s nucleotide, we examined time course experiments of lipid I syntheses with N-glycolyl Park’s nucleotide-Ne-dansylthiourea 1 and N-acyl Park’s nucleotide-Ne-dansylthiourea 2. MurX-catalyzed syntheses of lipid I analogues with 1 and 2 are summarized in Table 1. All reactions were per- formed in duplicate under the same reaction conditions except for the concentrations of decaprenyl phosphate and reaction time. The lipid I analogues 3 and 4 were chemically synthesized as reference molecules for HPLC studies [27,28]. Direct comparison of the product yields for 3 and 4 revealed that Park’s nucleotide-Ne-dansylthiourea 1 and 2 were converted into the corresponding lipid I analogues 3 and 4, respectively, by MurX without noticeable difference in reaction rate and product yield (entry 1 vs. 4 and entry 2 vs. 5 in Table 1). Increasing concentrations of decaprenyl phosphate (from 2 to 10 equivalents against 1 or 2) did not dra- matically increase the product yield (entry 3 vs. 6 in Table 1).

Kinetic studies revealed that N-glycolyl Park’s nucleotide-Ne-dansylthiourea 1 and N-acyl Park’s nucleotide-Ne-dansylthiourea 2 have a similar binding afﬁnity toward MurX with Km values of 18.05 and 17.95 lM, respectively [19]. Although Mtb predominantly uses N-glycolyl Park’s nucleotide for the biosynthesis of PG through N-glycolyl lipid I [13], MurX recognizes N-acetyl Park’s nucleotide equally. These structural tolerances of the Park’s nucleotide binding domain of MurX imply the possibility of further simpliﬁcation of Park’s nucleotide for development of a convenient MurX/MraY assay.

Mycobacterium spp. use decaprenyl phosphate for the biosyn- thesis of lipid I [15]. Due to the fact that decaprenyl-lipid I does not dissolve in water media, isolation and quantitation of the gen- erated lipid I in the assay reaction mixtures are time-consuming processes (see above). In our hands, it is extremely difﬁcult to develop medium- and high-throughput screening for MurX/MraY assay using decaprenyl phosphate. We accomplished a practical chemical synthesis of neryl-lipid I and its ﬂuorescent probes and characterized their physicochemical properties. Neryl-lipid I-Ne-dansylthiourea 5 showed excellent water solubility (>50 lg/ml), and 5 can readily be analyzed via reverse-phase HPLC without the gradient elution method (retention time: <9 min). MurX-cata- lyzed lipid I analogue synthesis with Park’s nucleotide-Ne-dansylthiourea 2 and neryl phosphate (5 equivalent against 2) in the presence of 0.5% Triton X-100 furnished neryl-lipid I-Ne-dansylthiourea 5 in 3 to 5% yield after 1 h [39]. Under the same reaction conditions, the yield of 5 was proportionated to the concentrations of neryl phosphate (6); the same reaction with 60 equivalents of neryl phosphate furnished 5 in greater than 50% yield in 1 h (Fig. 2B). As summarized in Fig. 2C, the effect of a phase transfer catalyst, Triton X-100, was observed in the transformation of 2 to 5 [41], where 0.5% Triton X-100 was determined to be the ultimate phase transfer catalyst of concentration for biosynthesis of lipid I-Ne-dansylthiourea analogue 5 with 2 and neryl phosphate (6). In short reaction times, application of higher concentrations of MurX enzyme (P-60) dramatically increased the reaction rate in biosynthesis of the lipid I analogue (Fig. 2D). However, Park’s nucleotide-Ne-dansylthiourea 2 could be transformed to the neryl lipid I analogue 5 in greater than 90% yield even at lower concen- trations of P-60 after 12 h (Fig. 2E). Among the other reaction parameters examined for MurX-catalyzed neryl-lipid I synthesis, the reaction temperatures did not noticeably affect the reaction rate within a temperature range between 22 and 37 oC. Thus, MurX assays can be performed conveniently at the ambient air tempera- tures. It is worth mentioning that all data obtained with P-60 membrane fraction from Mtb could be reproduced with that from *M. smegmatis*.

MurX/MraY assays

The MraY-catalyzed transformation of lipid I from Park’s nucle- otide is believed to be a reversible process [19,42]. On the contrary, under the optimized conditions, MurX-catalyzed neryl-lipid I synthesis from Park’s nucleotide-Ne-dansylthiourea 2 is not an equilibrium reaction; the 2 could be completely consumed to form 5 within 15 h (Fig. 2E). The neryl-lipid I analogue 5 can readily be dissolved in water or the assay media. Without extraction of the MurX/MraY enzymatic product from the reaction mixtures, the assay media can be assayed directly via reverse-phase HPLC for quantitation. Separation of Park’s nucleotide analogue 2 and neryl-lipid I analogue 5 could be performed via a C8 or C18 reverse-phase column with a ﬁxed solvent system (CH3CN/0.05 M aq. NH4HCO3 = 25:75) at a ﬂow rate of 0.5 ml/min. Under these assay conditions, the retention times of Park’s nucleotide 2 and neryl-lipid I analogue 5 were 4.0 and 7.9 min, respectively (Fig. 3). On the other hand, separation of Park’s nucleotide and decaprenyl-lipid I required a gradient method for HPLC analyses [19]; the retention time of decaprenyl-lipid I, 4 (Table 2), was 60 min under our optimized HPLC conditions (C18 reverse-phase column; solvent systems: MeOH/0.05 M aq. NH4HCO3 = 85:15 to 100% MeOH; ﬂow rate: 2.0 ml/min). The Km value for Park’s nucle- otide-dansylthiourea 2 was 18.29 lM at a concentration of 450 lM neryl phosphate; this was very similar to the Km value obtained with decaprenyl or undecaprenyl phosphate (Km = 18.05 lM) [19]. The Vmax for neryl-lipid I synthesis by MurX was determined to be 2.69 x 10-3 lM/s through the Michaelis–Menten plot. As stated above, a signiﬁcant difference in MurX/MraY-catalyzed neryl-lipid I synthesis is that the transformation from Park’s nucleotide 2 to neryl-lipid I 5 is not a reverse process and neryl-lipid I 5 is bio- synthesized in greater than 80% yield in 8 h (Fig. 2E). Although the synthesis of neryl-lipid I 5 could be achieved with Park’s nucleotide-Ne-dansylthiourea 2 in greater than 50% yield within 1 h via the puriﬁed MraY enzymes, unlike P-60 membrane fraction, the reactions did not attain greater than 65% yield even after 15 h, probably due to instability of the puriﬁed MraY under the assay reaction conditions. Thus, MurX/MraY assays have been conve- niently performed using P-60 membrane fractions obtained from Mtb or *M. smegmatis*.

The ﬂuorescence characteristics of the Ne-dansylthiourea moiety in the enzymatic substrate and product can be applied to ﬂuo- rescence-based analytical technics in order to quantitate the inhibition of lipid I biosynthesis. However, the signal-to-noise ratio of HPLC analyses using UV (350 nm) is excellent enough for quantitation of 10 ll of the assay mixtures. The range of linearity was established by injections (via an autosampler) of six concentrations of Park’s nucleotide-Ne-dansylthiourea 2 and neryl-lipid I-Ne-dansylthiourea 5 (r P 0.90), and the limit of detection was determined to be much lower than 1-lM concentrations.

Validation of MurX assays with antimycobacterial uridyl peptides

To determine the usefulness of the MurX assay developed here, we examined several known MraY inhibitor molecules and negative controls and demonstrated effectiveness of this MurX assay by screening our uridyl peptide library molecules. Known MraY inhibitors, capuramycin (7) and SQ641 (8), exhibited strong enzyme inhibitory activity against MurX (entries 1 and 2 in Table 2); the IC50 values of 6 and 7 were 0.152 and 0.109 lM, respectively [43]. Interestingly, these antimycobacterial MraY inhibitors 7 and 8 showed a 10-fold decrease in enzyme inhibitory activity against E. coli MraY. MurX enzyme inhibitory activity of tunicamycin (9) was determined to be the IC50 value of 2.73 lM, where the observed activity of 9 was closely related to the data reported in the literature (2.40–2.95 lM) [19]. In our screening of series of MraY inhibitors in enzyme and bacterial growth inhibi- tory assays, the MurX inhibitors that showed IC50 values above

10 lM did not exhibit signiﬁcant bactericidal activity against Mtb

[44–46]. Thus, we set up an IC50 threshold of 10 lM to distinguish

exploitable antimycobacterial MurX inhibitors from other antimy- cobacterial molecules. The analogues of capuramycin 10 and 11 have been used as negative controls in our program. These mole- cules did not show MurX inhibitory activity even at 100-lM con- centrations (entries 4 and 5 in Table 2). Although vancomycin

showed the good activity in MraY–MurG coupled assays, it could be conﬁrmed that vancomycin did not inhibit MurX even at 100- lM concentrations. We evaluated more than 50 synthetic uri- dine-glycosyl peptides and uridyl peptides that showed MIC values of less than 25 lg/ml against Mtb in the MurX assay screening at three to ﬁve different concentrations. Among the identiﬁed new MurX inhibitors, two molecules, 12 and 13, are worth highlighting. The 2’-methyl isomer 12 and amino-benzodiazepinone analogue 13 showed increased MurX inhibitory activity compared with capuramycin 10 (entries 7 and 8 in Table 2). On the other hand, the pacidamycin analogue 14 and muraymycin analogue 15 did not exhibit MurX inhibitory activity even at 100-lM concentrations [47,48]. Thus, we concluded that dihydrouridyl analogues 14 and 15 exhibited antimycobacterial activity by targeting the other essential enzyme(s) for growth of Mtb.

Therefore, the HPLC-based assay of MurX/MraY investigated here can be performed with standard analytical devices and will be adapted to medium- to high-throughput formats with close to ideal Z’ factors (0.5–1.0) [49]. The Z’ factor was estimated from the data summarized in Table 2; an estimated Z’ factor was 0.84; thus, the new MurX/MraY assay method described here is consid- ered to be an excellent assay. We are currently generating a rela- tively large number of library molecules containing known MurX/MraY inhibitors to examine robustness of the described assay method.

Conclusion

We have demonstrated MurX/MraY-catalyzed synthesis of neryl-lipid I-Ne-dansylthiourea 5 from Park’s nucleotide-Ne-dans- ylthiourea 2 with neryl phosphate (6). Biosynthesis of neryl-lipid I analogue 5 was achieved, for the ﬁrst time, in excellent yield with the MurX-containing membrane fraction (P-60) [50,51]. Similarly, neryl-lipid I-Ne-dansylthiourea 5 could be biosynthesized via the different sources of MraY enzymes such as *M. smegmatis*, *E. coli*, and *S. aureus*. However, the puriﬁed MraY enzymes seem to be denaturing under the assay conditions developed for P-60 mem- brane fractions. We are currently investigating the assay condi- tions that stabilize the puriﬁed MraY enzymes for the high-yield transformation from Park’s nucleotide-Ne-dansylthiourea 2 to neryl-lipid I-Ne-dansylthiourea 5. A water-soluble lipid I generated in MurX assay media could be quantitated conveniently via reverse-phase HPLC without sophisticated extraction procedures. The signal-to-noise ratio of HPLC analyses of 2 and 5 is signiﬁcantly high without using ﬂuorescence detector. Furthermore, the difference in retention times between Park’s nucleotide-Ne-dansylthiourea 2 and neryl-lipid I-Ne-dansylthiourea 5 was more than 3.5 min, and each assay analysis could be completed within 10 min. We developed convenient methods for preparation of MurX/MraY enzymatic substrates, Park’s nucleotide-Ne-dansylthiourea 2, and neryl phosphate (6); thus, the substrates for the assays are available to screen a relatively large number of molecules in our laboratory. To determine the usefulness of the MurX/MraY assay protocols developed here, we screened a 50-member library including new uridyl peptides and known positive and negative controls. MurX enzyme inhibitory activity of all positive controls showed IC50 values approximately equal to those obtained with the previously reported methods. The negative control molecules did not exhibit MurX inhibitory activity even at high concentra- tions. Because the assay method described here quantitates the MurX/MraY substrate and product simultaneously in each assay vial without extraction or separation, the errors in qualitative anal- yses of assay caused by quantitation of a single molecule (the remaining or converted molecule) and/or by complicated workup procedures are diminished. In the screening of a small library of molecules using the described method, to date a false positive or false negative result has not been identiﬁed. Some dihydropacid- amycins were reported to exhibit mycobacterial growth inhibitory activity in vitro [48]. However, their MurX enzyme inhibitory activities have not been thoroughly investigated. We observed an interesting trend in a series of (2R,5R)-(aminomethyl)-3-hydroxy- tetrahydrofuranyl)uridine derivatives; the dihydropacidamycin analogues (represented by 14 and 15) possessing antimycobacterial activity did not exhibit Mtb, *M. smegmatis*, and *E. coli* MraY enzyme inhibitory activities even at high concentrations. We have been studying the molecular target for antimycobacterial non- MurX/MraY inhibitors identiﬁed in this program. High reliability for the MurX/MraY assay protocols described here will be a vari- able asset to identify selective MurX inhibitor molecules for devel- opment of new antibacterial agents. Assay protocol and enzymatic substrates developed under this program will be provided to the scientiﬁc community.

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