



Published in final edited form as:

Anal Biochem. 2014 September 15; 461: 36–45. doi:10.1016/j.ab.2014.05.018.

## Biosynthesis of A Water-Soluble Lipid I Analogue and A Convenient Assay for Translocase I

Shajila Siricilla<sup>#1</sup>, Katsuhiko Mitachi<sup>#1</sup>, Karolina Skorupinska-Tudek<sup>2</sup>, Ewa Swiezewska<sup>2</sup>, and Michio Kurosu<sup>1,\*</sup>

<sup>1</sup>Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, 881 Madison Avenue, Memphis, TN 38163-0001, United States <sup>2</sup>Department of Lipid Biochemistry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warszawa, Poland

# These authors contributed equally to this work.

### 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 been received considerable attentions to the development of new 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 C<sub>50</sub>-prenyl unit that shows very poor water-solubility, and 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-*N*<sup>ε</sup>-dansylthiourea 2 that can be used as a MurX enzymatic substrate to form lipid I analogues. In our investigation of minimum structure requirement of the prenyl phosphate in the MraY/MurX-catalyzed lipid I analogue synthesis with 2, we found that neryl phosphate (C<sub>10</sub>-phosphate) can be recognized by MraY/MurX to generate the water-soluble lipid I analogue in quantitative yield under the optimized conditions. Herein, we report a rapid and robust analytical method for quantifying MraY/MurX inhibitory activity of library molecules.

### Keywords

Mur X; Mra Y; Translocase I; *Mycobacterium tuberculosis*; Water-soluble lipid I; Park's nucleotide; MraY assay; HTS; MraY inhibitors

---

The eradication of tuberculosis remains a prominent challenge for basic, translational, and clinical research scientists [1]. Once thought to be under control, tuberculosis case reports are increasing world-wide 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

---

\* Address correspondence to Michio Kurosu, mkurosu@uthsc.edu.

NIH-PA Author Manuscript  
NIH-PA Author Manuscript  
NIH-PA Author Manuscript

people died from TB [2-3]. One-third of the 42 million people living with HIV/AIDS worldwide are co-infected with Mtb [4-5]. Clinical responses of multidrug-resistant (MDR)-TB patients to the 1<sup>st</sup> line drugs have been poor, and in some cases there is no response at all. The WHO estimated that 650,000 new cases of MDR-TB emerge each year, and 27 countries around the world account for 86% of the MDR-TB burden. An outbreak of extensively-drug resistant (XDR)-Mtb was reported in 2006 [3,6]. For MDR strains of Mtb, treatment length of TB chemotherapy can be at least 20-28 months. The treatment of XDR-TB takes substantially longer than MDR-TB [4,7]. Thus, it is significantly important to discover promising approaches to shorten current TB drug regimen. In *in vitro* time-kill assessment experiments, FDA-approved TB drugs required 11 to 14 days to kill exponentially growing Mtb at 2-4×MIC concentrations. On the other hand, several translocase I (MraY/MurX, hereafter referred to as “MurX” for *Mtb* translocase I) inhibitors have been known to kill >95% of Mtb in 2-5 days at MIC or 2-4×MIC concentrations [8-9]. Since 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 *E. 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 orthologs have been identified in the Gram-positive genomes. However, very few genes responsible for the unique features of mycobacterial peptidoglycan 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 1) an *N*-glycolyl (NGlyc) in addition to *N*-acetyl (NAc) group on the muramic acid (Mur), 2) amidation of the carboxylic acids in the peptide moieties of PG, and 3) additional glycine or serine 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 unexploited drug targets. However, most of drugs associated with cell-wall biosynthesis may not reduce treatment time of a TB drug regimen because the dormant or non-replicating Mtb are not actively synthesizing 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 (C<sub>50</sub>) 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 that includes; 1) monitoring the transfer of phosphoryl-MurNAc-pentapeptide using fluorescent or radiolabeled Park’s nucleotide and/or undecaprenyl phosphate [19], 2) measuring the exchange reaction between [<sup>3</sup>H]UMP to Park’s nucleotide that requires separation of [<sup>3</sup>H]uridine after the treatment of alkaline phosphatase [20,21], 3) an indirect

assay using a coupled *MraY*-*MurG* that requires biotinylated Park's nucleotide and [<sup>14</sup>C]UDP-GlcNAc [22], 4) an assay using HP20ss hydrophobic beads for isolating the generated radiolabeled lipid I [23], 5) a microplate-based assay using a radiolabeled-Park's nucleotide [24], and 6) a scintillation proximity assay using wheat germ agglutinin-coated beads to capture the lipid I from a radiolabeled-Park's nucleotide [25]. Although a several assay methods were reported to be amenable to a 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 further optimization to be robust statistical methods that can identify *MraY*/*MurX* inhibitors routinely with IC<sub>50</sub> values. We established an efficient synthetic method for the generation of sufficient amount of fluorescent Park's nucleotide probes for HTS [27,28], and tested the Park's nucleotide probes in *MurX*-catalyzed lipid I analogue synthesis with decaprenyl and truncated prenyl phosphates. Surprisingly, under the optimized conditions the water-soluble lipid I-*neryl* (C<sub>10</sub>) analogue could be biosynthesized efficiently with the Park's nucleotide probes and *neryl* phosphate. In the present work, we report a convenient and reliable enzyme assay for *MurX* to identify antimycobacterial *MurX* inhibitor molecules.

## Materials and methods

### Chemical materials and methods

Difco Middlebrook 7H10 agar, Middlebrook 7H9 broth, Tryptic soy agar, Tryptic soy broth, MOPS, tris(hydroxymethyl)aminomethane, 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 Whatman 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 60F<sub>254</sub>) visualizing at 254 nm, or developed with ceric ammonium molybdate or anisaldehyde solutions by heating on a hot plate. <sup>1</sup>H-NMR spectral data were obtained using 400, and 500 MHz instruments. <sup>13</sup>C-NMR spectral data were obtained using 100 and 125 MHz instruments. For all NMR spectra, δ values are given in ppm and *J* values in Hz.

### *MurX*/*MraY* assay substrates

Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2**, *neryl*-lipid I-*N*<sup>ε</sup>-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 triethylamine (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 °C for 12h, and the reaction was cooled to room temperature. The reaction was quenched with water (5 mL) and stirred for 1h at 80 °C. The reaction mixture was cooled to room temperature, and the aqueous phase was extracted with

ether (5mL × 3). Lyophilization of the aqueous phase gave the crude product. Purification by DOWEX 50WX8 afforded neryl phosphate (**6**)-mono ammonium salt (0.17g, 74%) as a white solid [29]. This reaction could readily be scaled up to multi grams of neryl phosphate-mono ammonium salt. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 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); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 142.75, 133.95, 123.84, 120.62, 120.54, 61.88, 61.83, 31.18, 25.92, 24.81, 22.57, 16.91; <sup>31</sup>P NMR (162 MHz, D<sub>2</sub>O) δ 3.72; LRMS (EI) calcd for C<sub>10</sub>H<sub>20</sub>O<sub>4</sub>P (M+H<sup>+</sup>): 235.11, found: 235.05.

### Park's nucleotide-*N*<sup>ε</sup>-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 μmol) in 0.1 M aqueous NaHCO<sub>3</sub> solution (0.10 mL) was added 5-(dimethylamino)-*N*-(4-isothiocyanatophenyl)naphthalene-1-sulfonamide (3.0 mg, 7.8 μmol) in DMF (0.05 mL). After being stirred for 2.5h at room temperature, the reaction mixture was filtered. The filtrate was purified by reverse-phase HPLC [column: HYPERSIL GOLD™ (175 Å, 12 μm, 250 × 10 mm), solvents: a gradient elution of 0 : 100 to 30 : 70 CH<sub>3</sub>CN : 0.05 M aqueous NH<sub>4</sub>HCO<sub>3</sub> over 30 min, flow rate: 2.0 mL/min, UV: 350 nm] to afford **2** (2.4 mg, 60%, the retention time: 29 min). Similarly, *N*-acetyl Park's nucleotide (500 mg) was converted to **2** in 75% yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 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) calcd for C<sub>59</sub>H<sub>83</sub>N<sub>12</sub>O<sub>28</sub>P<sub>2</sub>S<sub>2</sub> (M+H<sup>+</sup>): 1533.44, found: 1533.80.

### Neryl Lipid I-*N*<sup>ε</sup>-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 Å, 12 μm, 250 × 10 mm). solvent: a gradient elution of 20 : 80 to 50 : 50 CH<sub>3</sub>CN : 0.05 M aqueous NH<sub>4</sub>HCO<sub>3</sub> over 30 min, flow rate: 2.0 mL/min, UV 350 nm] to afford **5** (2.0 mg, 70%, the retention time: 20 min). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 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) calcd for  $C_{60}H_{89}N_{10}O_{23}P_2S_2$  ( $M+H^+$ ): 1443.50, found: 1443.90.

### Bacterial strains and growth of bacteria

*Mycobacterium tuberculosis* (H37Rv) was obtained through BEI Resources, NIAID/NIH. *Mycobacterium smegmatis* (ATCC 607), *Staphylococcus aureus* (ATCC BAA-1556), and *Escherichia coli* K-12 (ATCC 29425) were obtained from ATCC. A single colony of bacterial strain was obtained on a Difco Middlebrook 7H10 nutrient agar enriched with 10% oleic acid, albumin, dextrose and catalase (OADC for *M. tuberculosis*), albumin, dextrose and catalase (ADC for *M. smegmatis*), and on Tryptic Soy agar (for *E. coli* and *S. aureus*). Seed cultures were obtained in Middlebrook 7H9 broth enriched with OADC (for *M. tuberculosis*), ADC (for *M. smegmatis*), and in Tryptic Soy broth (for *E. coli* and *S. aureus*), respectively. Each bacterium was grown to mid-log phase.

### Preparation of membrane fraction P-60 containing MurX/MraY

*M. tuberculosis* cells were harvested by centrifugation (4,700 RPM) at 4 °C followed by washing with 0.9% saline solution (thrice) and ~5g of pellet (wet weight) was collected. The washed cell pellets were suspended in homogenization buffer (containing 50 mM MOPS of pH = 8, 0.25 M sucrose, 10 mM  $MgCl_2$  and 5 mM 2-mercaptoethanol) and disrupted by probe sonication on ice (10 cycles of 60s on and 90s off). The resulting suspension was centrifuged at 1,000  $\times g$  for 10 min at 4 °C to remove unbroken cells. The supernatant was centrifuged at 25,000  $\times g$  for 40 min at 4 °C (3 to 4 times). All pellets in each tube were pooled and a second sonication was performed (10 cycles of 60s on and 90s off). The lysate was centrifuged once at 25,000  $\times g$  for 1h and the supernatant was subjected to ultracentrifugation at 60,000  $\times g$  for 1h at 4 °C. 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 are about 8~10 mg/mL [32]. Aliquots were stored in eppendorf tubes at -80°C. Similarly, the membrane fractions containing MraY enzyme (P-60) were prepared from *M. smegmatis*, *S. aureus*, and *E. coli*, respectively.

### MurX/MraY assay

Park's nucleotide-*N*<sup>c</sup>-dansylthiourea **2** (2 mM stock solution; 3.75  $\mu$ L (75  $\mu$ M)),  $MgCl_2$  (0.5 M; 10  $\mu$ L (50 mM)), KCl (2 M, 10  $\mu$ L (200 mM)), triton X100 (0.5%; 11.25  $\mu$ L), tris-buffer (pH = 8; 50mM, 2.5  $\mu$ L), neryl phosphate (**6**, 10 mM, 45  $\mu$ L), and inhibitor (0-100  $\mu$ M, in DMSO (2.5  $\mu$ L)) were placed in a 500  $\mu$ L Eppendorf tube. To a stirred reaction mixture, P-60 (15  $\mu$ L) was added (the total volume of the reaction mixture: 100  $\mu$ L). The reaction mixture was incubated for 1h at room temperature (26 °C), and quenched with  $CHCl_3$  (200  $\mu$ L). Two phases were mixed *via* vortex and centrifuged at 25,000  $\times g$  for 10 min. The upper aqueous phase was assayed *via* reverse-phase HPLC. The water phase (10  $\mu$ L) was injected into HPLC (solvent:  $CH_3CN:0.05$  M aq.  $NH_4HCO_3 = 25 : 75$ , UV: 350 nm, flow rate: 0.5 mL/min, Column: Kinetex 5u C<sub>8</sub> 100Å, 150  $\times$  4.60mm), and the area of the peak for lipid I-

neryl derivative **5** was quantified to obtain the IC<sub>50</sub> value. The IC<sub>50</sub> values were calculated from plots of the percent product inhibition versus the inhibitor concentration.

### Kinetic parameter evaluation *via* MurX/MraY activity assay

Evaluation of kinetic parameters were performed through MurX- or *M. smegmatis* MraY-catalyzed lipid I synthesis. K<sub>m</sub> and V<sub>max</sub> 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 the concentration of 1 mg/100 µL (stock solution). The MurX assays developed here were tolerated to 2.5% of DMSO concentrations in total volume of the reaction solution (100 µL). The maximum tolerated concentration for DMSO in MurX-catalyzed neryl lipid I-*N*<sup>ε</sup>-dansylthiourea **5** has not been determined.

### Determination of MICs

*M. tuberculosis* was cultured to be an optical density of 0.4-0.5. Each compound (8 µL) stored in DMSO (1 mg/100 µL) was placed in a sterile 96 well plate and a serial dilution was conducted with the culturing broth (total volume of 100 µL). The bacterial suspension (100 µL) was added to each well (total volume of 200 µL). The bacterial culture in a plate treated or non-treated with compounds was incubated for 14 days at 37 °C in a shaking incubator (120 rpm). Resazurin (0.01%, 20 µL) was added to each well and incubated at 37 °C for 5h. The MIC values were determined according to NCCLS method (pink = growth, blue = no visible growth). The absorbance of each well was also measured at 570 nm and 600 nm *via* a microplate reader.

### Results and discussion

A number of selective MraY inhibitors from natural sources have been reported [35-41]. The major source of future developments resides in the nucleoside based inhibitors, which are subdivided into 5 classes. Tunicamycin is a relatively weak MurX inhibitor [26], and did not show strong antimycobacterial activity. Caprazamycin, muraymycins, liposidomycin, capuramycin, mureidomycins, pacidamycins and their congeners were reported to exhibit significant MraY enzyme inhibitory activity *in vitro* [35]. Capuramycin showed activity specific to *Mycobacterium spp.* Antimicrobial spectrum focused against Mtb (selective antimycobacterial 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 resistant to other bacteria during TB chemotherapy. Most of the reported MraY inhibitors were competitive 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.* [40], we selected positive and negative controls from capuramycin derivatives to develop a convenient MurX assay method, and the new MurX assay



developed here was planned to demonstrate by screen antimycobacterial uridyl-peptide library molecules.

*N*-Glycolylated muramic (MurNGlyc) acid predominates in *M. tuberculosis* and MurNGlyc is the major muramyl building blocks in mycobacterial cell walls (Fig 1) [13,41]. Thus, it is of importance to characterize specificity of MurX against *N*-glycolyl Park's nucleotide and *N*-acetyl Park's nucleotide for discovery of novel antimycobacterial MurX inhibitors. In order 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 FITC-conjugated *N*-acetyl Park's nucleotides can be applied to *MraY* enzyme assays with the *MraY* obtained from Gram-negative and some Gram-positive organisms [41]. 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 5-25% yields after 1h incubation [31]. These results were in accordance with the data previously reported by several other groups [19,20]. It was experimentally proved that *MurX* was also tolerated in the structure of the C6-position (*N*<sup>ε</sup>) of lysine moiety of Park's nucleotides.

In order 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-*N*<sup>ε</sup>-dansylthiourea **1** and *N*-acyl Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2**. *MurX*-catalyzed syntheses of lipid I analogues with **1** and **2** are summarized in Table 1. All reactions were performed 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-*N*<sup>ε</sup>-dansylthiourea **1** and **2** were converted to 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 2 vs. 5 in Table 1). Increasing concentration of decaprenyl phosphate (from 2 to 10 equivalents against **1** or **2**) did not dramatically increase the product yield (Entry 3 vs. 6 in Table 1). Kinetic studies revealed that *N*-glycolyl Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **1** and *N*-acyl Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2** have a similar binding affinity towards *MurX* with the *K<sub>m</sub>* values of 18.05 and 17.95 μM, respectively [19]. Although *Mtb* predominantly uses *N*-glycolyl Park's nucleotide for the biosynthesis of peptidoglycan 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* implies the possibility of further simplification of Park's nucleotide for development of a convenient *MurX*/*MraY* assay.

*Mycobacterium spp.* uses decaprenyl phosphate (C<sub>50</sub>-P) for the biosynthesis of lipid I [15]. Due to the fact that C<sub>50</sub>-lipid I does not dissolve in water media, thus, isolation and quantitation of the generated lipid I in the assay reaction mixtures are time-consuming processes (*vide supra*). In our hands, it is extremely difficult to develop medium- and high-throughput screening (HTS) 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-*N*<sup>H</sup>-dansylthiourea **5** showed excellent water-solubility (>50 µg/mL) and **5** can readily be analyzed *via* reverse-phase HPLC without gradient elution method (retention time: <9 min.) (Fig 3). MurX-catalyzed lipid I analog synthesis with Park's nucleotide-*N*<sup>E</sup>-dansylthiourea **2** and neryl phosphate (5 equivalent against **2**) in the presence of 0.5% of triton × furnished neryl-lipid I-*N*<sup>E</sup>-dansylthiourea **5** in 3-5% yield after 1h [40]. 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 1h (Fig 2B). As summarized in Fig 2C, effect of a phase transfer catalyst, triton X100 was observed in the transformation of **2** to **5** [42]; 0.5% of triton X100 was determined to be the ultimate phase transfer catalyst of concentration for biosynthesis of lipid I-*N*<sup>E</sup>-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-*N*<sup>E</sup>-dansylthiourea **2** could be transformed to the neryl lipid I analogue **5** in greater than 90% yield even at lower concentrations of P-60 after 12h (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 °C. Thus, MurX assays can be performed conveniently at the ambient air temperatures. 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 nucleotide is believed to be a reversible process [19,43]. On the contrary, under the optimized conditions MurX-catalyzed neryl-lipid I synthesis from Park's nucleotide-*N*<sup>E</sup>-dansylthiourea **2** is not an equilibrium reaction; **2** could be completely consumed to form **5** within 15h (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 C<sub>8</sub>- or C<sub>18</sub>-reverse phase column with a fixed solvent system (CH<sub>3</sub>CN : 0.05 M aq. NH<sub>4</sub>HCO<sub>3</sub> = 25 : 75) at flow rate of 0.5 mL/min. Under these assay conditions, the retention times of Park's nucleotide **2** and neryl-lipid I **5** were 4.0 min. 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 (C<sub>18</sub>-reverse phase column, solvent systems: (MeOH : 0.05 M aq. NH<sub>4</sub>HCO<sub>3</sub> = 85 : 15 to 100% MeOH, flow rate: 2.0 mL/min). The *K<sub>m</sub>* value for Park's nucleotide-dansylthiourea **2** was 18.29 µM at the concentrations of 450 µM of neryl phosphate; this was very similar to the *K<sub>m</sub>* values obtained with decaprenyl or undecaprenyl phosphate (*K<sub>m</sub>*: 18.05 µM) [19]. The *V<sub>max</sub>* for neryl-lipid I synthesis by MurX was determined to be 2.69 × 10<sup>-3</sup> µM/sec through the Michaelis-Menten plot. As stated above, 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 biosynthesized in



greater than 80% yield in 8h (Fig 2E). Although the synthesis of neryl-lipid I **5** could be achieved with Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2** in over 50% yield within 1h *via* the purified *MraY* enzymes, unlike P-60 membrane fraction, the reactions did not attain over 65% yield even after 15h due probably to instability of the purified *MraY* under the assay reaction conditions. Thus, *MurX*/*MraY* assays have been conveniently performed using P-60 membrane fractions obtained from *Mtb* or *M. smegmatis*.

The fluorescence characteristics of the *N*<sup>ε</sup>-dansylthiourea moiety in the enzymatic substrate and product can be applied to fluorescence-based analytical techniques in order to quantitate the inhibition of lipid I biosynthesis. However, signal-to-noise ratio of HPLC analyses using UV (350 nm) is excellent enough for quantitation of 10 μL of the assay mixtures. The range of linearity was established by injections (*via* an auto sampler) of six concentrations of Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2** and neryl-lipid I-*N*<sup>ε</sup>-dansylthiourea **5** ( $r = 0.9$ ), and limit of detection was determined to be much lower than 1 μM concentrations.

### Validation of *MurX* assays with antimycobacterial uridyl peptides

In order to determine 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  $IC_{50}$  values of **6** and **7** were 0.152 and 0.109 μM, respectively [45]. 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  $IC_{50}$  value of 2.73 μM in which the observed activity of **9** was closely related to the data reported in the literatures (2.40-2.95 μM) [19]. In our screening of series of *MraY* inhibitors in enzyme and bacterial growth inhibitory assays, the *MurX* inhibitors that showed the  $IC_{50}$  value of above 10 μM did not exhibit significant bactericidal activity against *Mtb* [46-48]. Thus, we set up an  $IC_{50}$  threshold of 10 μM to distinguish exploitable antimycobacterial *MurX* inhibitors from other antimycobacterial molecules. The analogues of capuramycin **10** and **11** have been used as negative controls in our program. These molecules did not show *MurX* inhibitory activity even at 100 μM concentrations (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 μM concentrations. We evaluated over 50 synthetic uridine-glycosyl peptides and uridyl peptides that showed the MIC values of <25 μg/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 worthwhile highlighting. The 2'-methyl isomer **12** and amino-benzodiazepinone analogue **13** showed increased *MurX* inhibitory activity compared to capuramycin **10** (entry 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 μM concentrations [49-50]. Thus, we concluded that dihydrouridyl analogues **14** and **15** exhibited antimycobacterial activity by targeting the other essential enzyme(s) for growth of *Mtb*.

Thus, 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) [44]. The  $Z'$ -factor was estimated from the data summarized in Table 2; an estimated  $Z'$ -factor was 0.84, and thus, the new MurX/MraY assay method described here is considered to be an excellent assay. We are currently generating a relatively 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- $N^{\epsilon}$ -dansylthiourea **5** from Park's nucleotide- $N^{\epsilon}$ -dansylthiourea **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) [51-52]. Similarly, neryl-lipid I- $N^{\epsilon}$ -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 membrane fractions. We are currently investigating the assay conditions that stabilize the purified MraY enzymes for the high-yield transformation from Park's nucleotide- $N^{\epsilon}$ -dansylthiourea **2** to neryl-lipid I- $N^{\epsilon}$ -dansylthiourea **5**. A water-soluble lipid I generated in MurX assay media could be quantitated conveniently *via* reverse-phase HPLC without sophisticated extraction procedures. Signal-to-noise ratio of HPLC analyses of **2** and **5** is significantly high without using fluorescence detector. Furthermore, difference in the retention times between Park's nucleotide- $N^{\epsilon}$ -dansylthiourea **2** and neryl-lipid I- $N^{\epsilon}$ -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- $N^{\epsilon}$ -dansylthiourea **2** and neryl phosphate (**6**), and thus, the substrates for the assays are available to screen a relatively large number of molecules in our laboratory. In order to determine usefulness of the MurX/MraY assay protocols developed here, we screened a 50-membered library including new uridyl peptides, and known positive- and negative-controls. MurX enzyme inhibitory activity of all positive-controls showed approximately equal to the  $IC_{50}$  values obtained with the previously reported methods. The negative-control molecules did not exhibit MurX inhibitory activity even at high concentrations. 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 analyses of assay caused by quantitation of single-molecule (remaining molecule or converted molecule) and/or by complicated work-up 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 dihydropacidamycins were reported to exhibit mycobacterial growth inhibitory activity *in vitro* [50]. However, their MurX enzyme inhibitory activities have not been thoroughly investigated. We observed an interesting trend in a series of (2*R*, 5*R*)-(aminomethyl)-3-hydroxytetrahydrofuran-yl)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 variable asset to identify selective MurX inhibitor molecules for development of new antibacterial agents. Assay protocol and enzymatic substrates developed under this program will be provided to the scientific community.

## Acknowledgments

The National Institutes of Health is greatly acknowledged for financial support of this work (AI084411). We also thank University of Tennessee for generous financial support. NMR data were obtained on instruments supported by the NIH Shared Instrumentation Grant. The following reagent was obtained through BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv and Gamma-Irradiated *Mycobacterium tuberculosis*, NR-14819. The authors gratefully acknowledge Drs. William Clemons (California Institute Technology) and Crick (Colorado State University) for useful discussions.

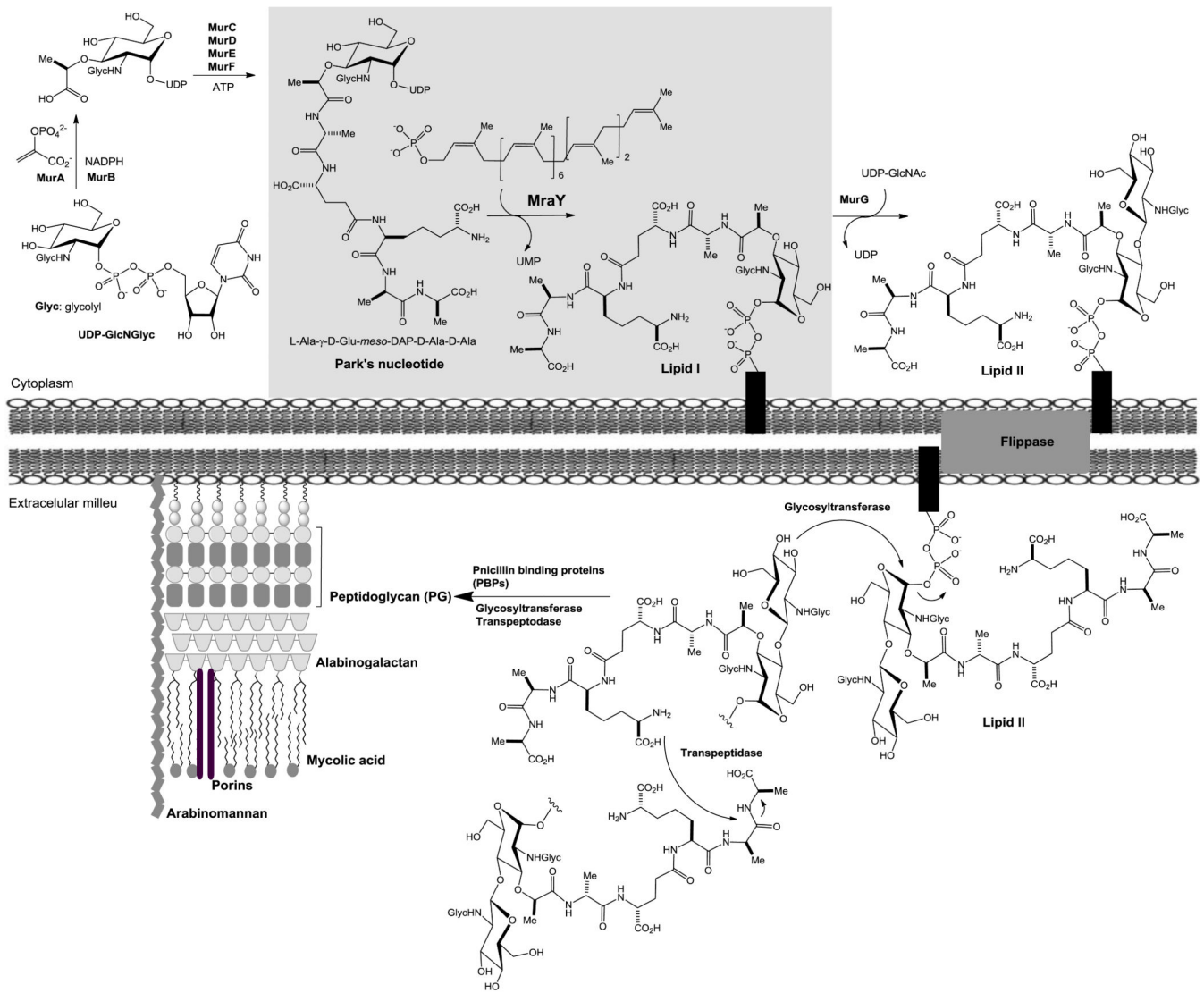
## References

- [1]. Stover CK, Warrener P, VanDevater DR, Sherman DR, Arain TM, Langhorne MH, Anderson SW, Towell JA, Yuan Y, McMurray DN, Kreiswirth BN, Barry CE, Baker WR. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature*. 2000; 405:962–966. [PubMed: 10879539]
- [2]. Lamichhanea G, Freundlich JS, Ekins S, Wickramaratnea N, Nolana ST, Bishaia WR. Essential metabolites of *Mycobacterium tuberculosis* and their mimics. *mBio*. 2011; 2:1–10.
- [3]. Chien JY, Lai CC, Tan CK, Huang YT, Chou CH, Hung CC, Yang PC, Hsueh PR. Decline in rates of acquired multidrug-resistant tuberculosis after implementation of the directly observed therapy, short course (DOTS) and DOTS-Plus programmes in Taiwan. *J. Antimicrob. Chemother.* 2013; 68:1910–1916. [PubMed: 23580558]
- [4]. Connolly LE, Edelstein PH, Ramakrishnan L. Why is long-term therapy required to cure tuberculosis? *PLoS Med.* 2007; 4:435–442.
- [5]. Dworkin J, Shah IM. Exit from dormancy in microbial organisms. *Nat. Rev. Microbiol.* 2010; 8:890–896. [PubMed: 20972452]
- [6]. Portero JL, Rubio M. New anti-tuberculosis therapies. *Expert Opin. Ther. Pat.* 2007; 17:617–637.
- [7]. Miranda MS, Breiman A, Allain S, Deknuydt F, Altare F. The tuberculous granuloma: An unsuccessful host defense mechanism providing a safety shelter for the bacteria? *Clin. Dev. Immunol.* 2012:1–14.
- [8]. Reddy VM, Einck L, Nacy CA. In Vitro antimycobacterial activities of capuramycin analogues. *Antimicrob. Agents Chemother.* 2008; 52:719–721. [PubMed: 18070956]
- [9]. Nikonenko BV, Reddy VM, Protopopova M, Bogatcheva E, Einck L, Nacy CA. Activity of SQ641, a capuramycin analog, in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 2009; 53:3138–3139. [PubMed: 19414567]
- [10]. Auger G, van Heijenoort J, Mengin-Lecreux D, Blanot D. A MurG assay which utilizes a synthetic analogue of lipid I. *FEMS Microbiol. Lett.* 2003; 219:115–119. [PubMed: 12594032]
- [11]. van Heijenoort J. Lipid intermediates in the biosynthesis of bacterial peptidoglycan. *Microbiol. Mol. Biol. Rev.* 2007; 71:620–635. [PubMed: 18063720]
- [12]. Bupp K, van Heijenoort J. The final step of peptidoglycan subunit assembly in *Escherichia coli* occurs in the cytoplasm. *J. Bacteriol.* 1993; 175:1841–1843. [PubMed: 8449890]
- [13]. Raymond JB, Mahapatra S, Crick DC, Pavelka MS. Identification of the namH gene, encoding the hydroxylase responsible for the N-glycosylation of the Mycobacterial peptidoglycan. *J. Biol. Chem.* 2005; 280:326–333. [PubMed: 15522883]
- [14]. Mahapatra S, Scherman H, Brennan PJ, Crick DC. N-Glycosylation of the nucleotide precursors of peptidoglycan biosynthesis of *Mycobacterium* spp. is altered by drug treatment. *J. Bacteriol.* 2005; 187:2341–2347. [PubMed: 15774877]

- [15]. Mahapatra S, Crick DC, Brennan PJ. Comparison of the UDP-N-acetylmuramate:L-alanine ligase enzymes from *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *J. Bacteriol.* 2000; 182:6827–6830. [PubMed: 11073931]
- [16]. Barry CE, Blanchard JS. The chemical biology of new drugs in development for tuberculosis. *Curr. Opin. Chem. Biol.* 2010; 14:456–466. [PubMed: 20452813]
- [17]. Kurosu M, Mahapatra S, Narayanasamy P, Crick DC. Chemoenzymatic synthesis of park's nucleotide: toward the development of high-throughput screening for *MraY* inhibitors. *Tetrahedron Lett.* 2007; 48:799–803.
- [18]. Timothy DH, Lloyd AJ, Roper DI. Phospho-MurNAc-pentapeptide translocase (*MraY*) as a target for antibacterial agents and antibacterial proteins. *Infect. Disord.: Drug Targets.* 2006; 6:85–106. [PubMed: 16789873]
- [19]. Stachyra T, Dini C, Ferrari P, Bouhss A, van Heijenoort J, Mengin-Lecreulx D, Blanot D, Biton J, Le Beller D. Fluorescence detection-based functional assay for high-throughput screening for *MraY*. *Antimicrob. Agents Chemother.* 2004; 48:897–902. [PubMed: 14982781]
- [20]. Weppner WA, Neuhaus FC. Fluorescent substrate for nascent peptidoglycan synthesis. Uridine diphosphate-N-acetylmuramyl-(N-epsilon-5-dimethylaminonaphthalene-1-sulfonyl)pentapeptide. *J. Biol. Chem.* 1977; 252:2296–2303. [PubMed: 849930]
- [21]. Geis A, Plapp R. Phospho-N-acetylmuramoyl-pentapeptide-transferase of *Escherichia coli* K12. Properties of the membrane-bound and the extracted and partially purified enzyme. *Biochim. Biophys. Acta.* 1978; 527:414–424. [PubMed: 215212]
- [22]. Branstorma AA, Midha S, Longley CB, Han K, Baizman ER. Assay for identification of inhibitors for bacterial *MraY* translocase and *MurG* transferase. *Anal. Biochem.* 2000; 280:315–319. [PubMed: 10790316]
- [23]. Hyland SA, Anderson MS. A high-throughput solid-phase extraction assay capable of measuring diverse polyphenyl phosphate: sugar-1-phosphate transferases as exemplified by *WecA*, *MraY* and *MurG* proteins. *Anal. Biochem.* 2003; 317:156–164. [PubMed: 12758253]
- [24]. Solapure SM, Raphael P, Gayathri CN, Barde SP, Chandrakala B, Das KS, deSousa SM. Development of a microplate-based scintillation proximity assay for *MraY* using a modified substrate. *J. Biomol. Screening.* 2005; 10:149–156.
- [25]. Ravishankar S, Prasanna Kumar V, Chandrakala B, Jha RK, Solapure SM, deSousa SM. Scintillation proximity assay for inhibitors of *Escherichia coli* *MurG* and, optionally, *MraY*. *Antimicrob. Agents Chemother.* 2005; 49:1410–1418. [PubMed: 15793120]
- [26]. Shapiro AB, Jahic H, Gao N, Hajec L, Rivin O. A high-throughput, homogeneous, fluorescence resonance energy transfer-based assay for phospho-N-acetylmuramoyl-pentapeptide translocase (*MraY*). *J. Biomol. Screening.* 2012; 17:662–672.
- [27]. Li K, Kurosu M. Synthetic studies on *Mycobacterium tuberculosis* specific fluorescent park's nucleotide probe. *Heterocycles.* 2008; 76:455–469.
- [28]. Mitachi K, Mohan P, Siricilla S, Kurosu M. One-pot protection-glycosylation reactions for synthesis of lipid II analogues. *Chem. - Eur. J.* 2014; 20:1–6.
- [29]. Dueymes C, Pirat C, Pascal R. Facile synthesis of simple mono-alkyl phosphates from phosphoric acid and alcohols. *Tetrahedron Lett.* 2008; 49:5300–5301.
- [30]. Rezwan M, Laneelle MA, Sander P, Daffe M. Breaking down the wall: Fractionation of mycobacteria. *J. Microbiol. Methods.* 2007; 68:2–39.
- [31]. Wolfe LM, Mahaffey SB, Kruh NA, Dobos KM. Proteomic definition of the cell wall of *Mycobacterium tuberculosis*. *J. Proteome Res.* 2010; 9:5816–5826. [PubMed: 20825248]
- [32]. Bouhss A, Crouvoisier M, Blanot D, Mengin-Lecreulx D. Purification and characterization of the bacterial *MraY* translocase catalyzing the first membrane step of peptidoglycan biosynthesis. *J. Biol. Chem.* 2004; 279:29974–29980. [PubMed: 15131133]
- [33]. Ha S, Chang E, Lo MC, Men H, Park P, Ge M, Walker S. The kinetic characterization of *Escherichia coli* *MurG* using synthetic substrate analogues. *J. Am. Chem. Soc.* 1999; 37:8415–8426.
- [34]. Ma Y, Münch D, Schneider T, Sahl HG, Bouhss A, Ghoshdastider U, Wang J, Dötsch V, Wang X, Bernhard F. Preparative scale cell-free production and quality optimization of *MraY*

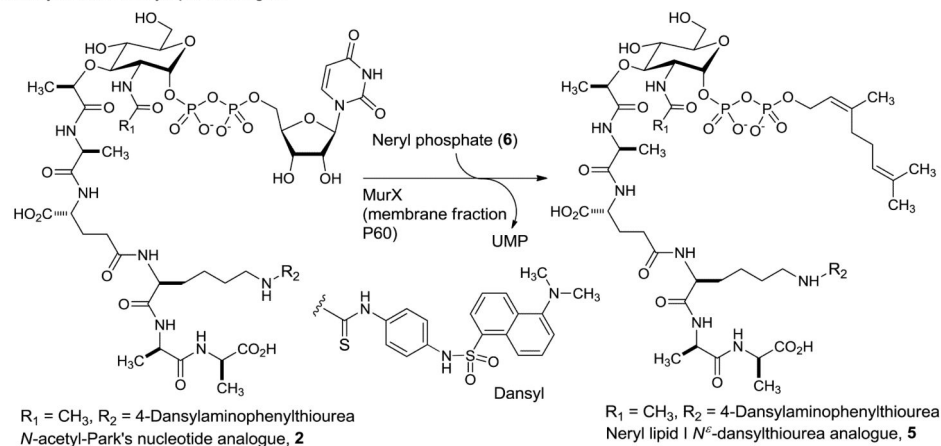
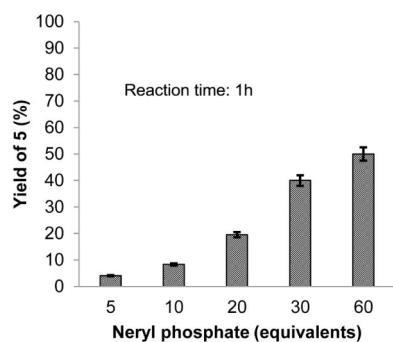
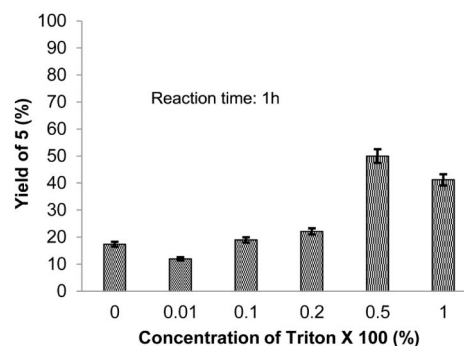
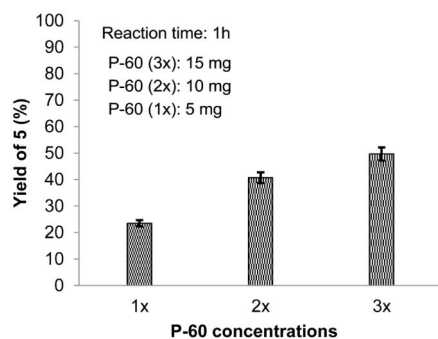
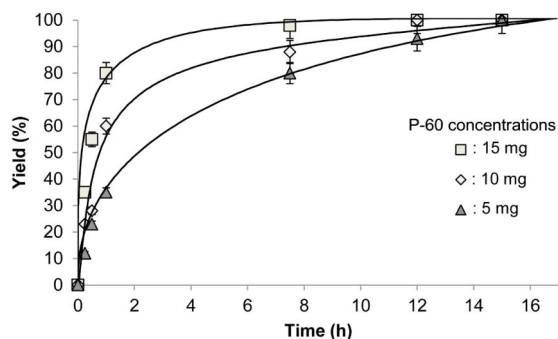
- homologues in different expression modes. *J. Biol. Chem.* 2011; 286:38844–38853. [PubMed: 21937437]
- [35]. Timothy DH, Lloyd AJ, Roper DI. Phospho-MurNAc-pentapeptide translocase (MraY) as a target for antibacterial agents and antibacterial proteins. *Infect. Disord. : Drug Targets.* 2006; 6:85–106. [PubMed: 16789873]
- [36]. Winn M, Goss RJM, Kimura K, Bugg TDH. Antimicrobial nucleoside antibiotics targeting cell wall assembly: Recent advances in structure–function studies and nucleoside biosynthesis. *Nat. Prod. Rep.* 2009; 27:279–304. [PubMed: 20111805]
- [37]. Yamashita A, Norton E, Petersen PJ, Rasmussen BA, Singh G, Yang Y, Mansour TS, Ho DM. Muraymycins, novel peptidoglycan biosynthesis inhibitors: synthesis and SAR of their analogues. *Bioorg. Med. Chem. Lett.* 2003; 13:3345–3350. [PubMed: 12951123]
- [38]. Boojamra CG, Lemoine RC, Lee JC, Leger R, Stein KA, Vernier NG, Magon A, Lemovskaya O, Martin PK, Chamberland S, Lee MD, Hecker SJ, Lee VJ. Stereochemical elucidation and total synthesis of dihydropacidamycin D, a semisynthetic acidamycin. *J. Am. Chem. Soc.* 2001; 123:870–874. [PubMed: 11456620]
- [39]. Dini C. MraY inhibitors as novel antibacterial agents. *Curr. Top. Med. Chem.* 2005; 5:1221–1236. [PubMed: 16305528]
- [40]. Kurosu M, Li K. Synthetic studies towards the identification of novel capuramycin analogs with antimycobacterial activity. *Heterocycles.* 2009; 77:217–225.
- [41]. Chen KT, Kuan YC, Fu WC, Liang PH, Cheng TJR, Wong CH, Cheng WG. Rapid preparation of mycobacterium *N*-Glycolyl lipid I and lipid II derivatives: A biocatalytic approach. *Chem. - Eur. J.* 2013; 19:834–838. [PubMed: 23229320]
- [42]. Brandish PE, Burnham MK, Lonsdale JT, Southgate R, Inukai M, Bugg TDH. Slow binding inhibition of phospho-*N*-acetylmuramyl-pentapeptide-translocase (*Escherichia coli*) by Mureidomycin A. *J. Biol. Chem.* 1996; 271:7609–7614. [PubMed: 8631795]
- [43]. Nuhaus FC. Initial translocation reaction in biosynthesis of peptidoglycan by bacterial membranes. *Acc. Chem. Res.* 1971; 4:297–303.
- [44]. Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screening.* 1999; 4:67–73.
- [45]. Koga T, Fukuoka T, Harasaki T, Inoue H, Hotoda H, Kakuta M, Muramatsu Y, Yamamura N, Hoshi M, Hirota T. Activity of capuramycin analogs against *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium intracellulare* in vitro and in vivo. *J. Antimicrob. Chemother.* 2004; 54:755–760. [PubMed: 15347635]
- [46]. Wang Y, Siricilla S, Alewi BA, Kurosu M. Improved synthesis of capuramycin and its analogues. *Chem. - Eur. J.* 2013; 19:13847–13858. [PubMed: 24014478]
- [47]. Kurosu M, Li K, Crick DC. A concise synthesis of capuramycin. *Org. Lett.* 2009; 11:2393–2396. [PubMed: 19405507]
- [48]. Alewi BA, Schneider CM, Kurosu M. Synthesis of ureido-muraymycidine derivatives for structure activity relationship studies of muraymycins. *J. Org. Chem.* 2012; 77:3859–3867. [PubMed: 22458337]
- [49]. Boojamra CG, Lemoine RC, Blais J, Vernier NG, Stein KA, Magon A, Chamberland S, Hecker SJ, Lee VJ. Synthetic dihydropacidamycin antibiotics: A modified spectrum of activity for the acidamycin class. *Bioorg. Med. Chem. Lett.* 2003; 13:3305–3309. [PubMed: 12951115]
- [50]. Lin YI, Li Z, Francisco GD, McDonald LA, Davis RA, Singh G, Yang Y, Mansour TS. Muraymycins, novel peptidoglycan biosynthesis inhibitors: semisynthesis and SAR of their derivatives. *Bioorg. Med. Chem. Lett.* 2002; 12:2341–1344. [PubMed: 12161129]
- [51]. Boonjamra CG, Lemoine RC, Blais J, Venier NG, Stein KA, Magon A, Chamberland S, Hecker SJ, Lee VJ. Synthetic dihydropacidamycin antibiotics: A modified spectrum of activity for the acidamycin class. *Bioorg. Med. Chem. Lett.* 2003; 13:3305–3309. [PubMed: 12951115]
- [52]. Breukink E, van Heusden HE, Vollmerhaus PJ, Swiezewska E, Brunner L, Walker S, Heck AJR, de Kruijff B. Lipid II as an intrinsic component of the pore induced by nisin in bacterial membranes. *J. Biol. Chem.* 2003; 278:19898–19903. [PubMed: 12663672]
- [53]. Men H, Park P, Ge M, Walker S. Substrate synthesis and activity assay for MurG. *J. Am. Chem. Soc.* 1998; 120:2484–2485.



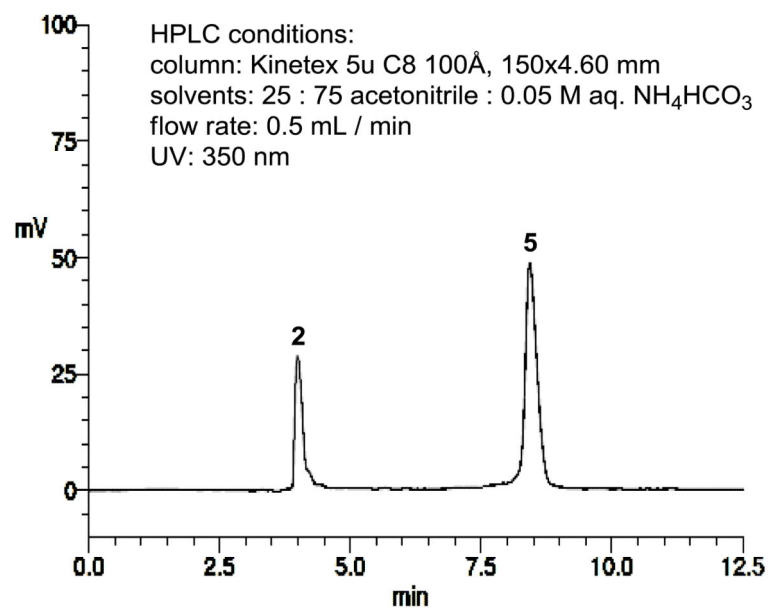


**Fig 1.**  
Biosynthesis of peptidoglycan in *Mycobacterium tuberculosis*.



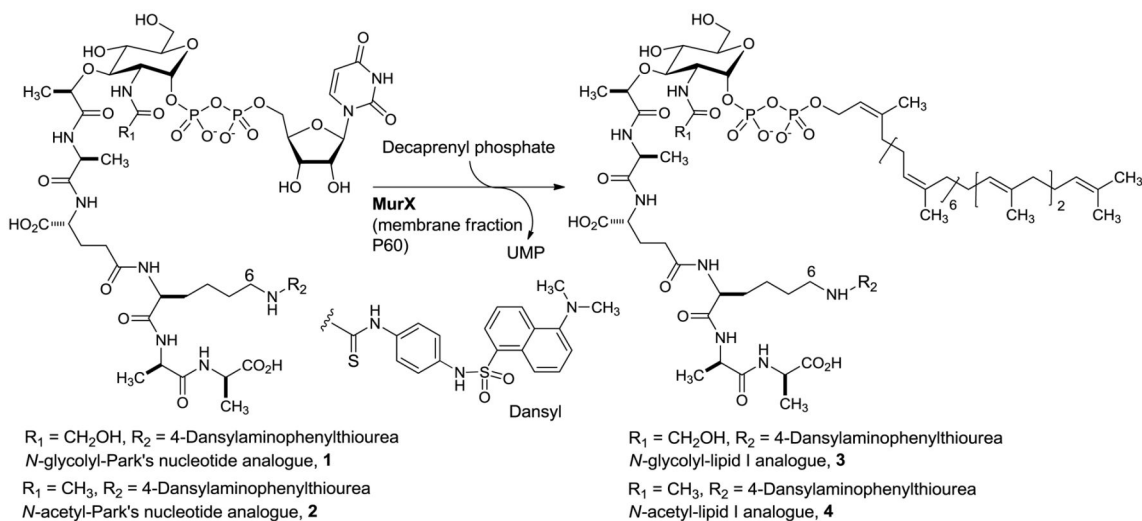
**A. Biosynthesis of neryl lipid I analogue.****B. Effect of concentrations of neryl phosphate in biosynthesis of 5.****C. Effect of triton X100 in biosynthesis of 5.****D. Effect of concentrations of P-60 in biosynthesis of 5.****E. Time-course experiments of neryl-lipid I syntheses.****Fig 2.**MurX-catalyzed biosynthesis of neryl lipid I analog **5**.

**A:** biosynthesis of neryl lipid I analogue **5** from Park's nucleotide analogue **2** and neryl phosphate **6**, **B:** effect of concentrations of neryl phosphate in biosynthesis of **5**, **C:** effect of a phase-transfer catalyst, triton x100 in biosynthesis of **5**, **D:** effect of concentrations of MurX-containing membrane fraction (P-60), **E:** time-course experiments of neryl-lipid I synthesis.



**Fig 3.**  
HPLC chromatogram of Park's nucleotide-*N*<sup>ε</sup>-dansyl analogue **2** and neryl lipid I-*N*<sup>ε</sup>-dansyl analogue **5**.

Table 1

MurX-catalyzed syntheses of lipid I analogues from *N*-glycolyl- and *N*-acetyl-Park's nucleotides

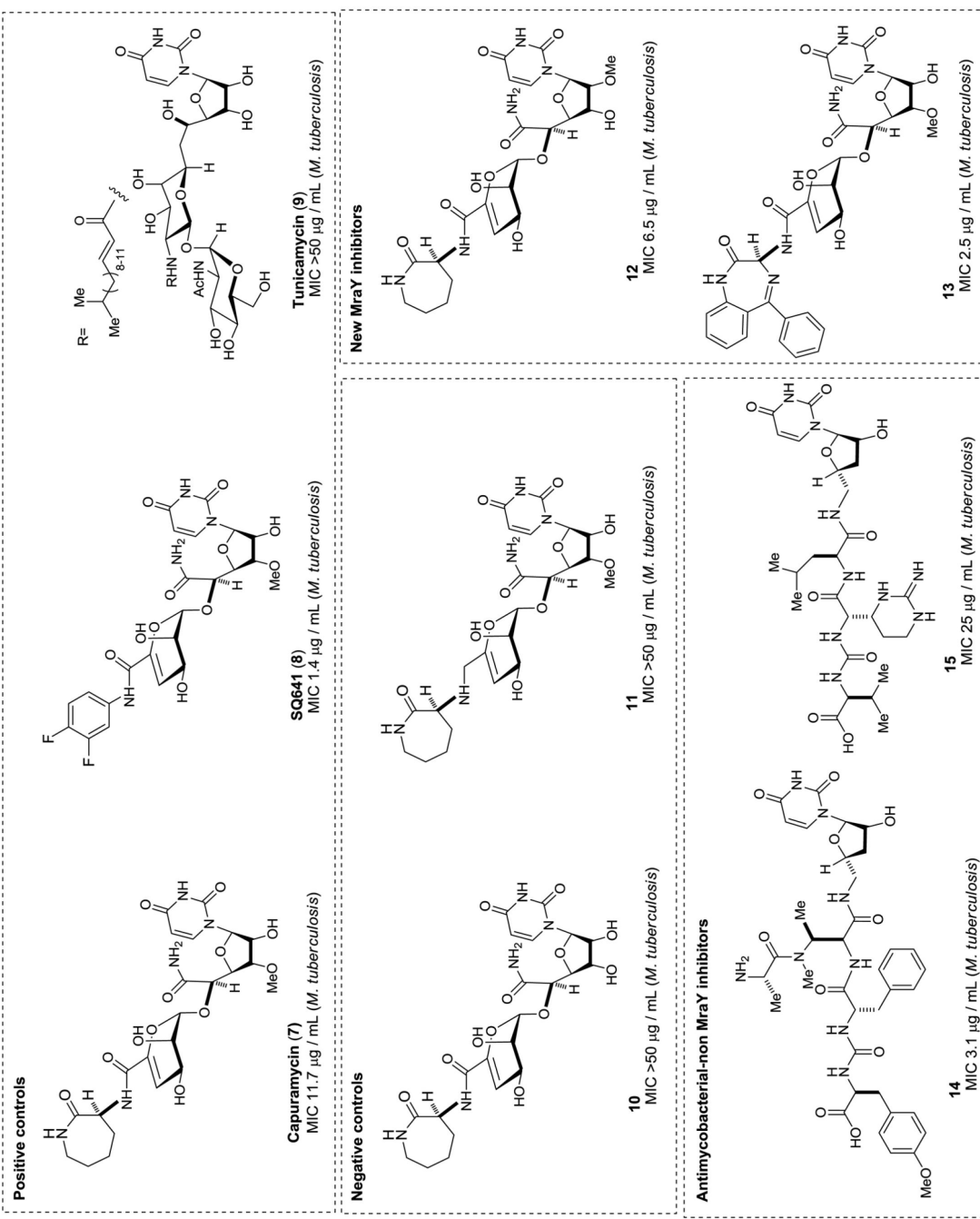
Entry <sup>1</sup>	Park's nucleotide	Decaprenyl phosphate (equivalents against 1 or 2)	Lipid I	Reaction time (h)	Yield (%) <sup>2</sup>
1	<b>1</b>	2	<b>3</b>	1	15-20
2	<b>1</b>	2	<b>3</b>	3	15-20
3	<b>1</b>	10	<b>3</b>	1	20-25
4	<b>2</b>	2	<b>4</b>	1	15-20
5	<b>2</b>	2	<b>4</b>	3	15-25
6	<b>2</b>	10	<b>4</b>	1	20-25

<sup>1</sup> Reaction conditions: Park's nucleotide (2 mM; 3.75  $\mu\text{L}$ ),  $\text{MgCl}_2$  (0.5 M; 10  $\mu\text{L}$ ); KCl (2 M, 10  $\mu\text{L}$ ), Triton X100 (0.1%; 11.25  $\mu\text{L}$ ), Tris-buffer (pH = 8; 50mM, 5  $\mu\text{L}$ ), decaprenyl phosphate (10 mM, 2 or 10 equivalents against 1 or 2), P-60 (15  $\mu\text{L}$ ), 26 °C, 1 or 3h.;

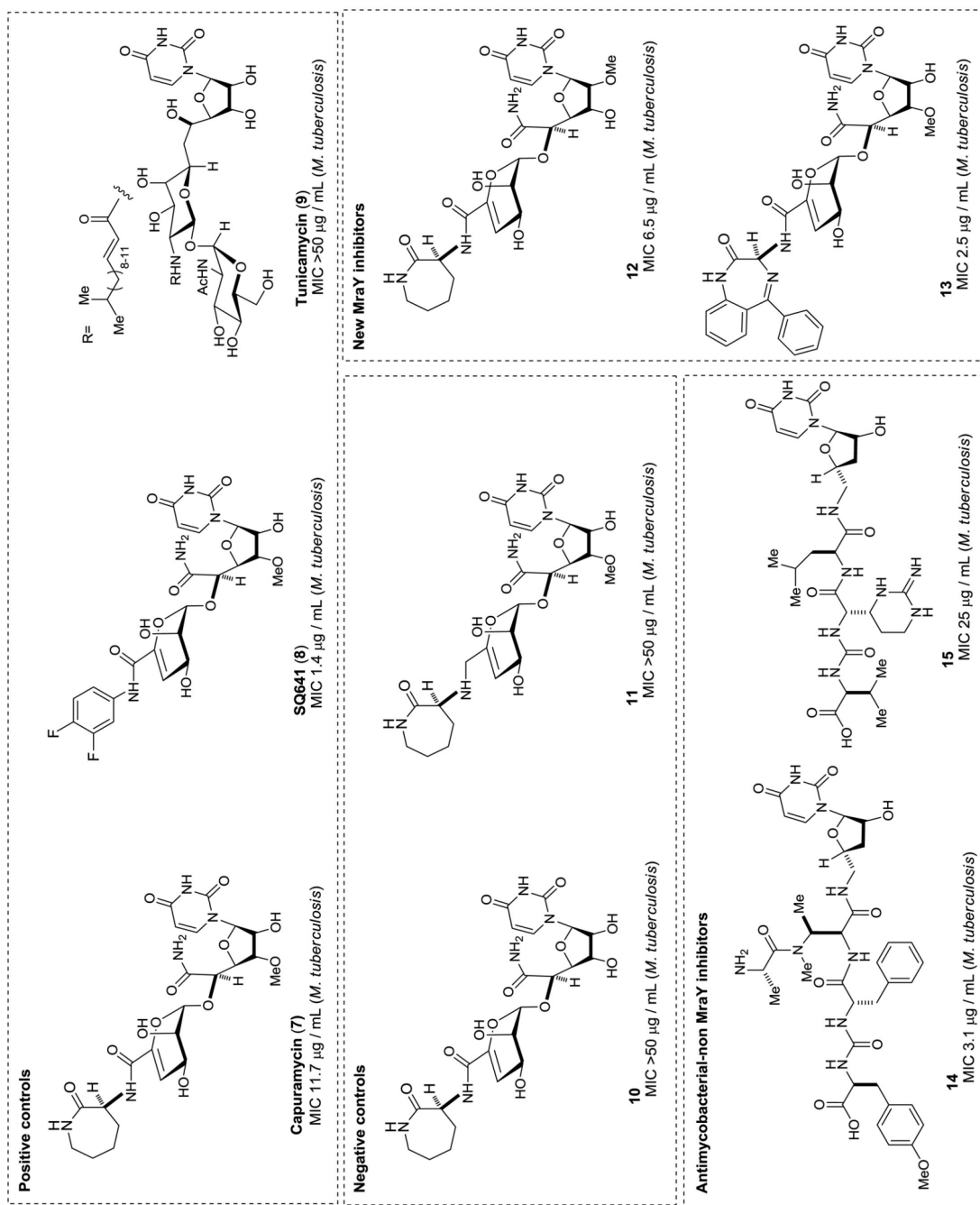
<sup>2</sup> n-butanol extract was analyzed by HPLC (column: Kinetex 5u C8 100A, 150 $\times$ 4.60 mm, solvent:  $\text{CH}_3\text{CN} : 0.05 \text{ M aq. NH}_4\text{HCO}_4$ , flow rate: 0.5 mL / min.

**Table 2**

Assay of positive- and negative-controls, and a library of uridylyl peptides against MurX

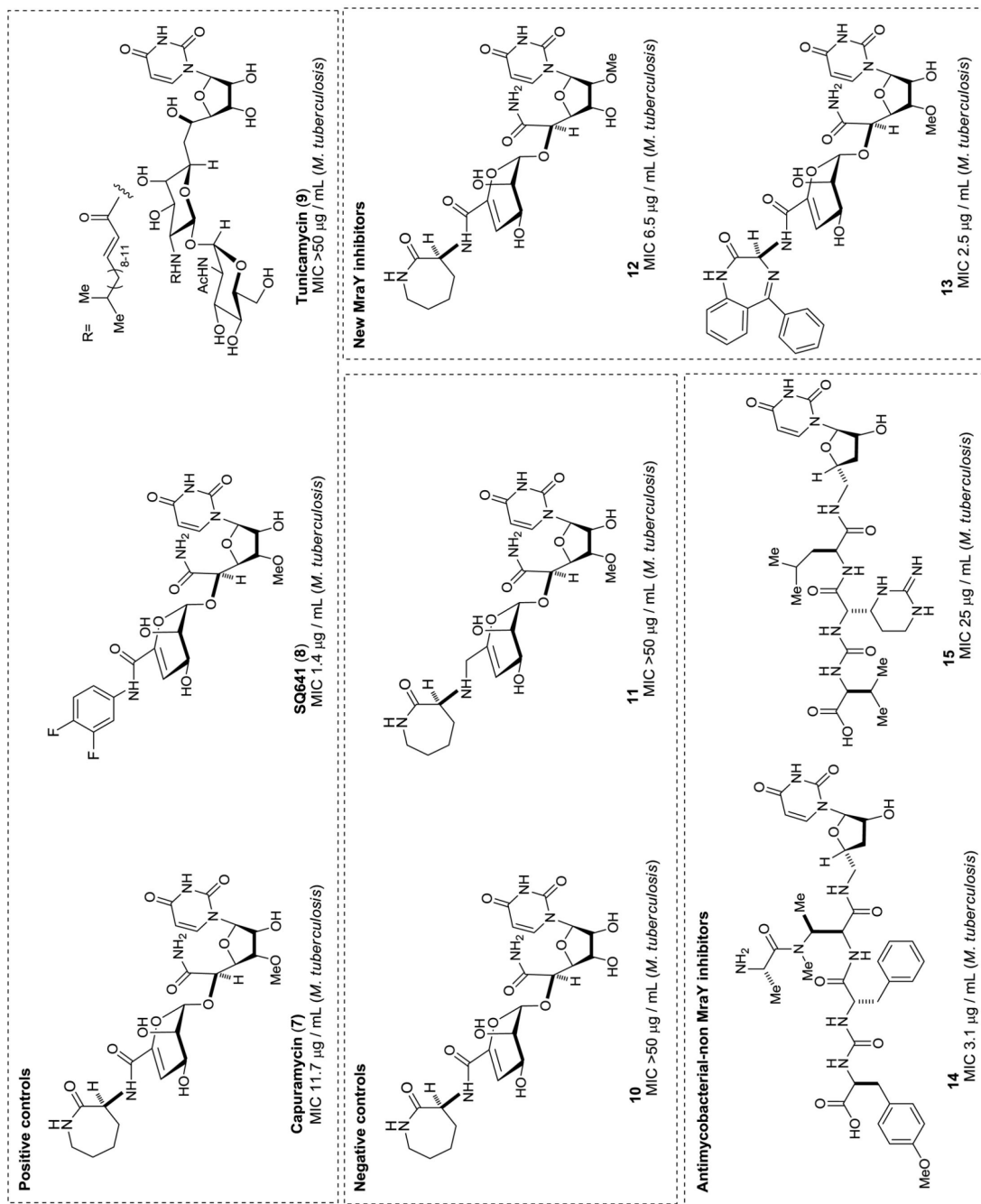


entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
1	Capuramycin (7)	-	36.6	88.4	100	100	0.152 $\pm$ 0.0125 (0.150-0.0180) <sup>c</sup>

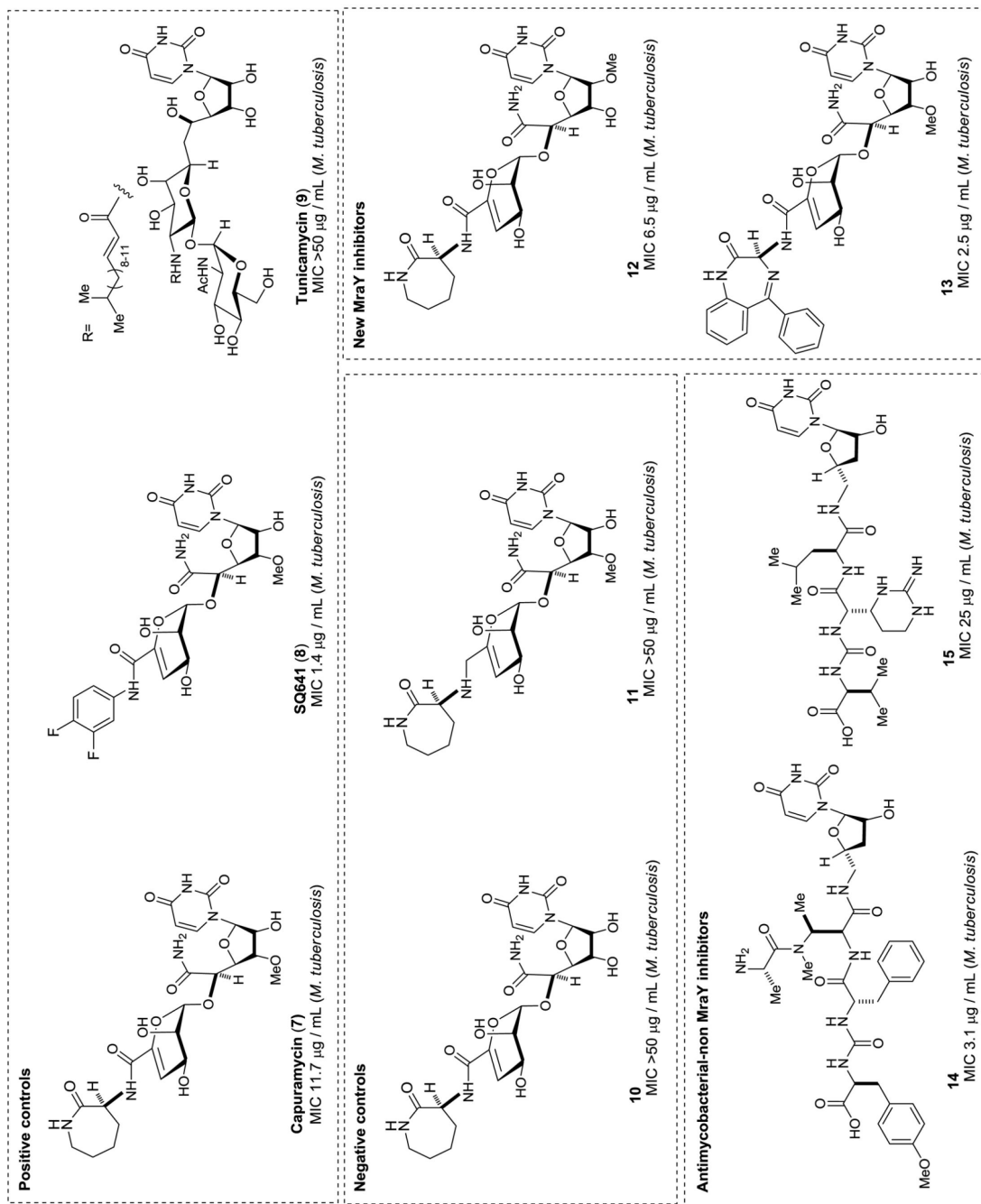


entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
2	SQ641 (8)	-	40	95	100	-	0.109 $\pm$ 0.00845 (0.0150-0.100) <sup>c</sup>

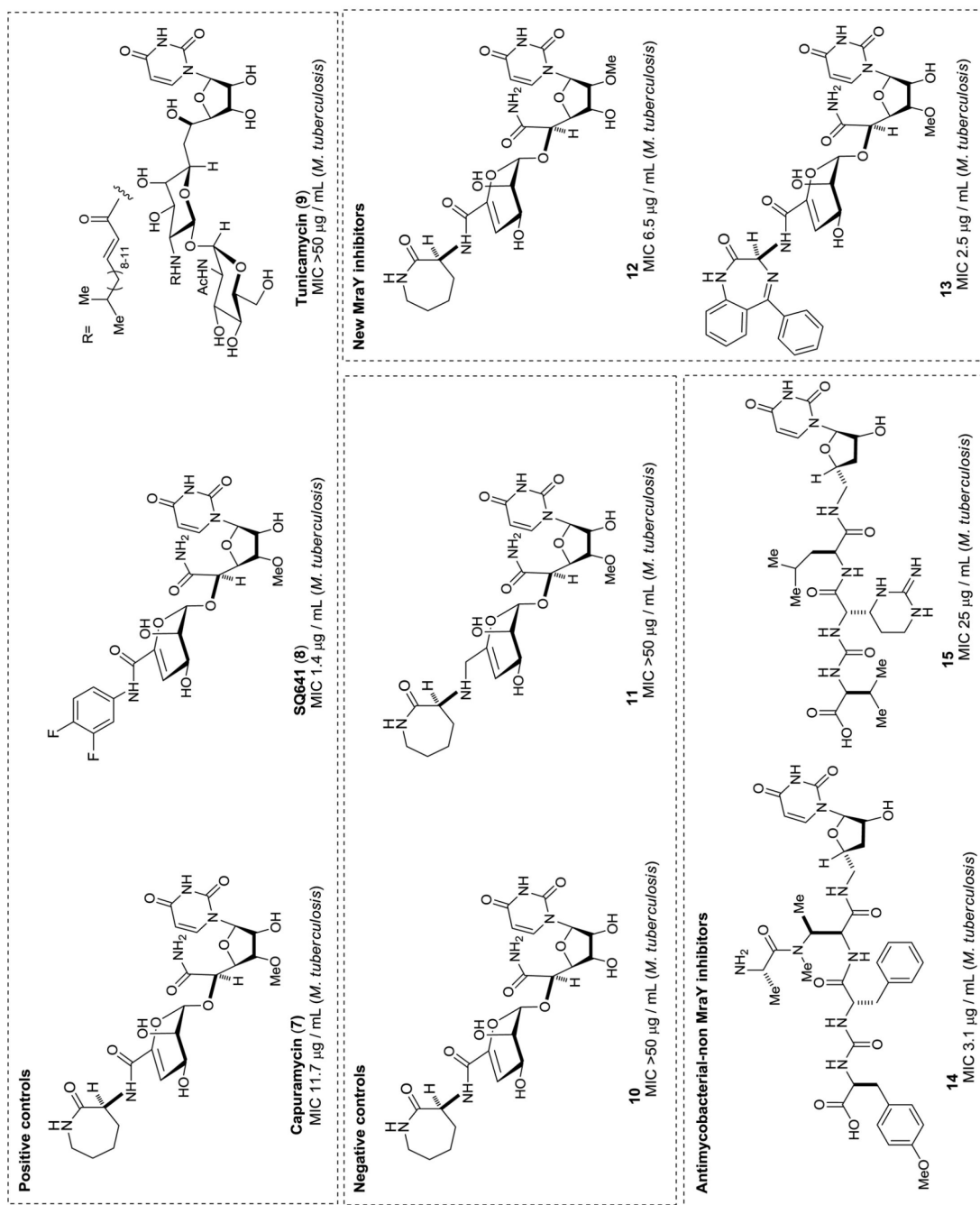




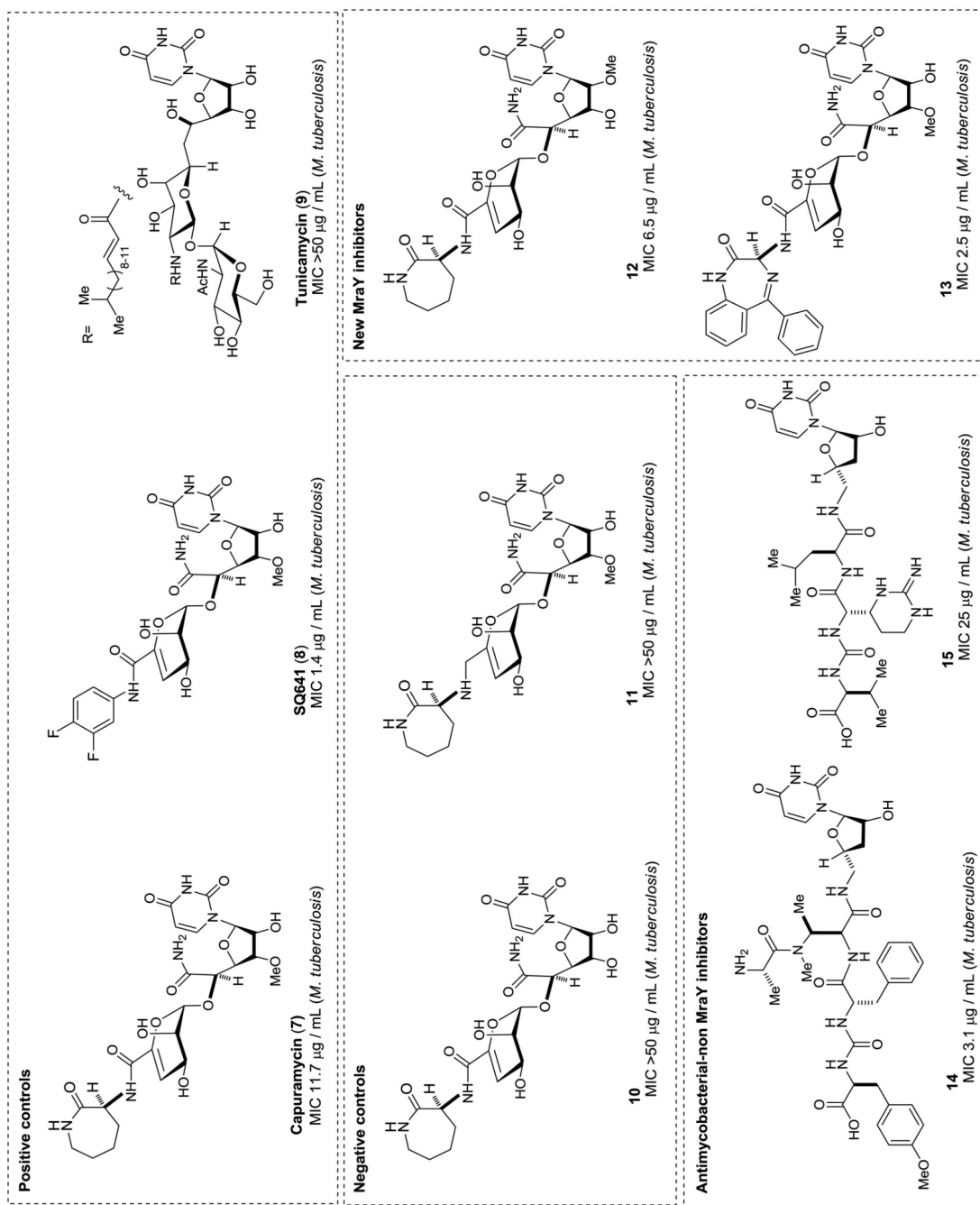
entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
3	Tunicamycin (9)	-	9	42	72	72	2.73 $\pm$ 0.138 (2.40-2.95) <sup>c</sup>



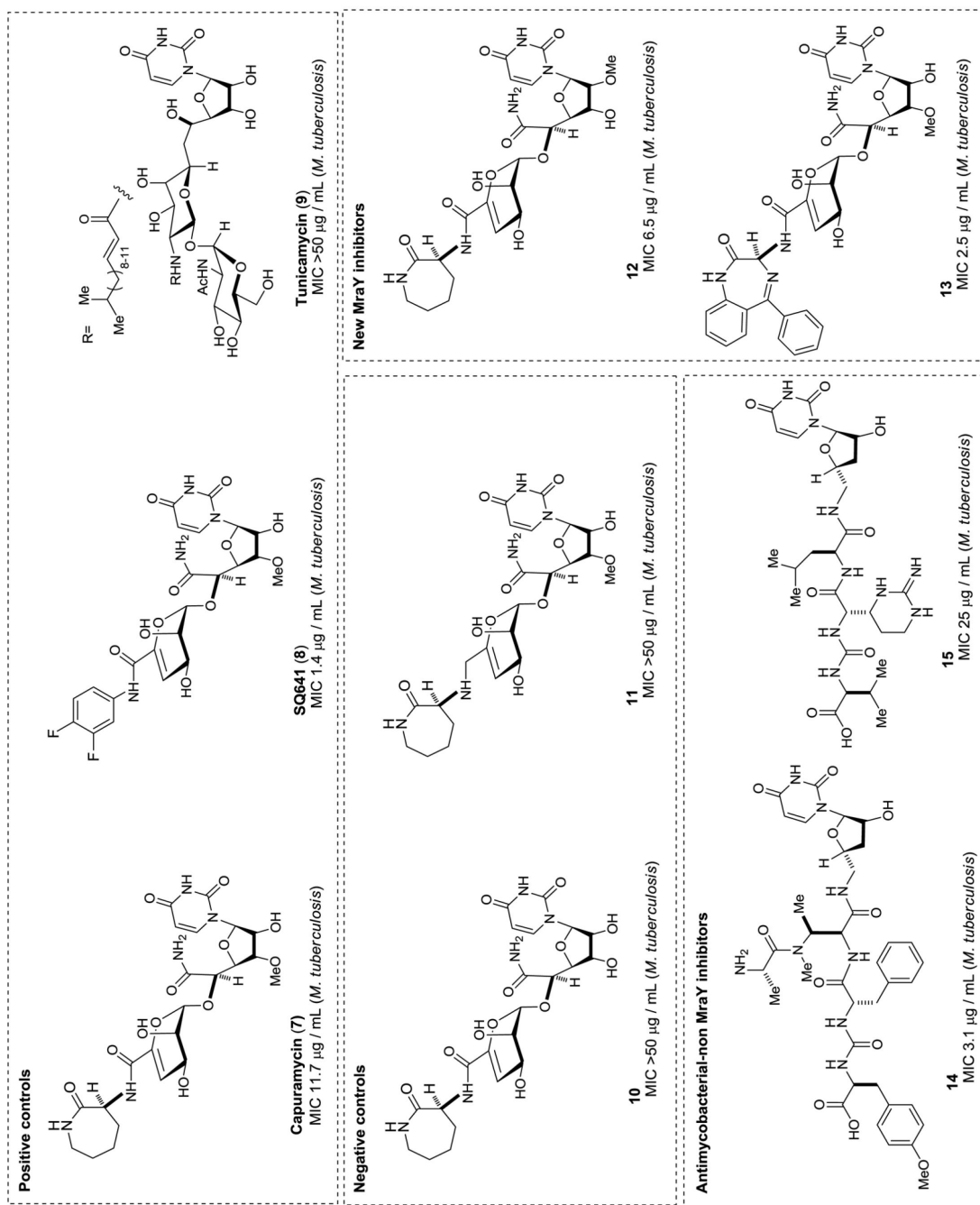
entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
4	10	-	0	0	0	0	ND



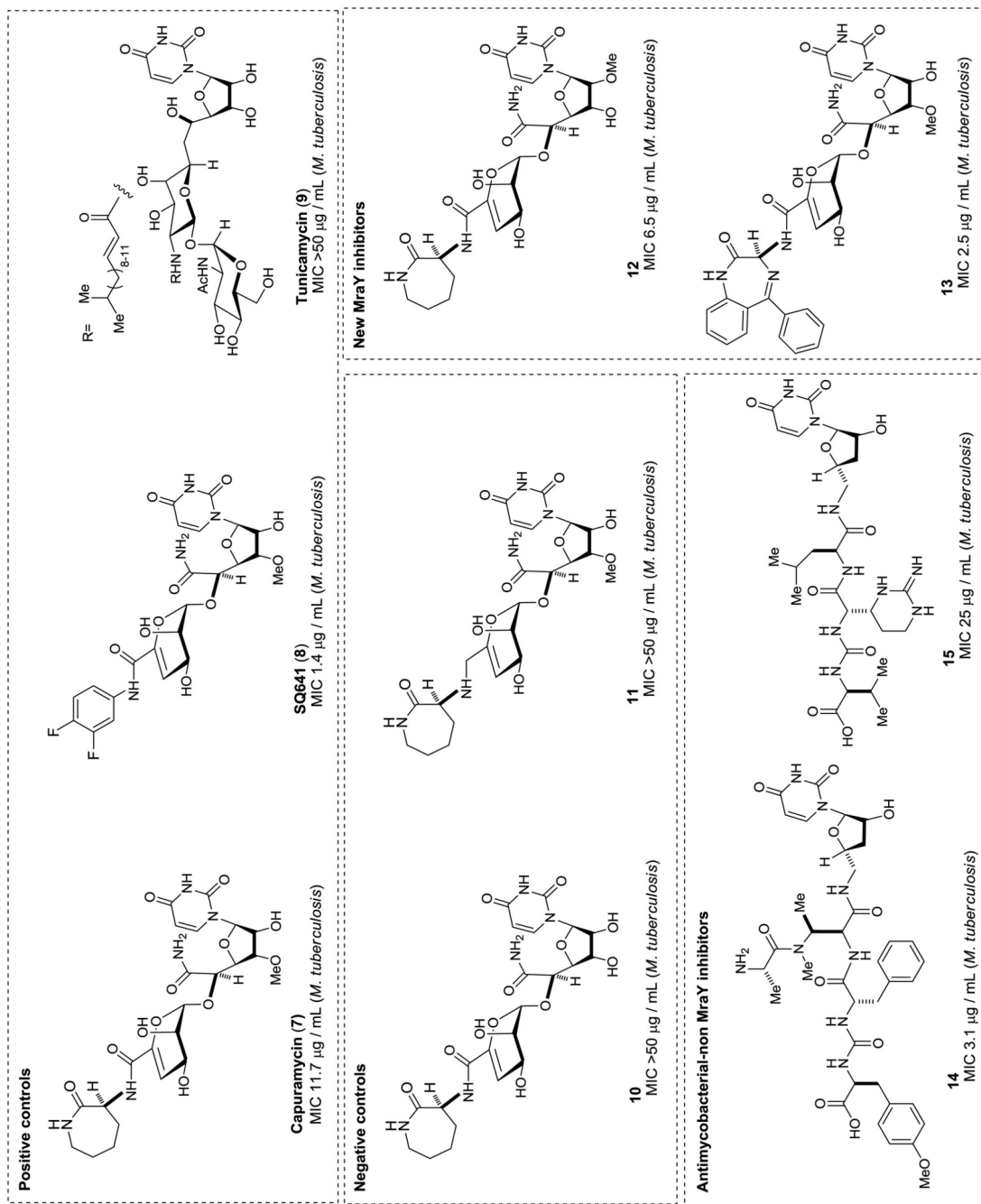
entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
5	11	-	0	0	0	0	ND



entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
6	Vancomycin	-	0	0	0	0	ND

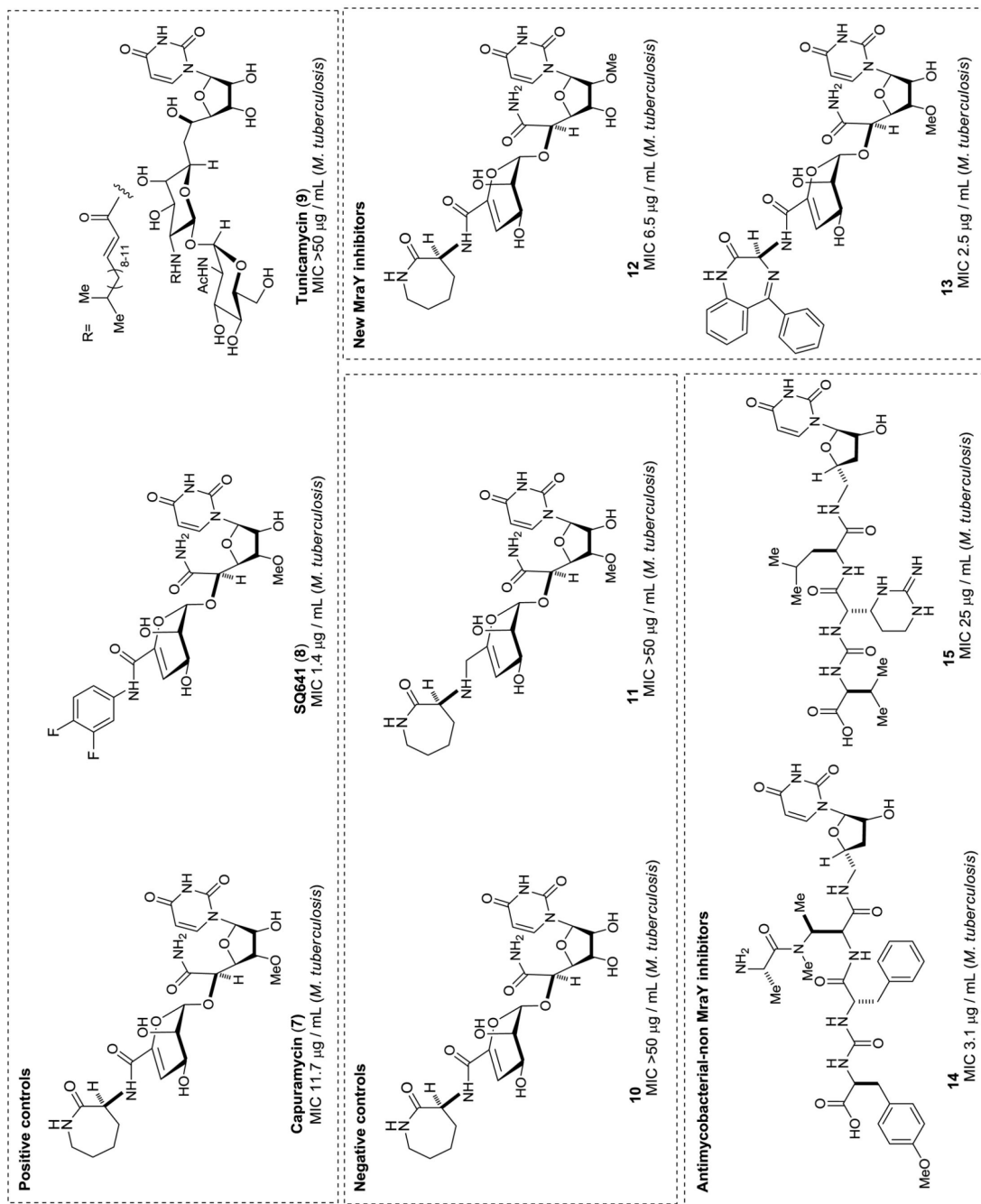


entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
7	12	3.8	81.2	100	100	-	0.105 $\pm$ 0.00330

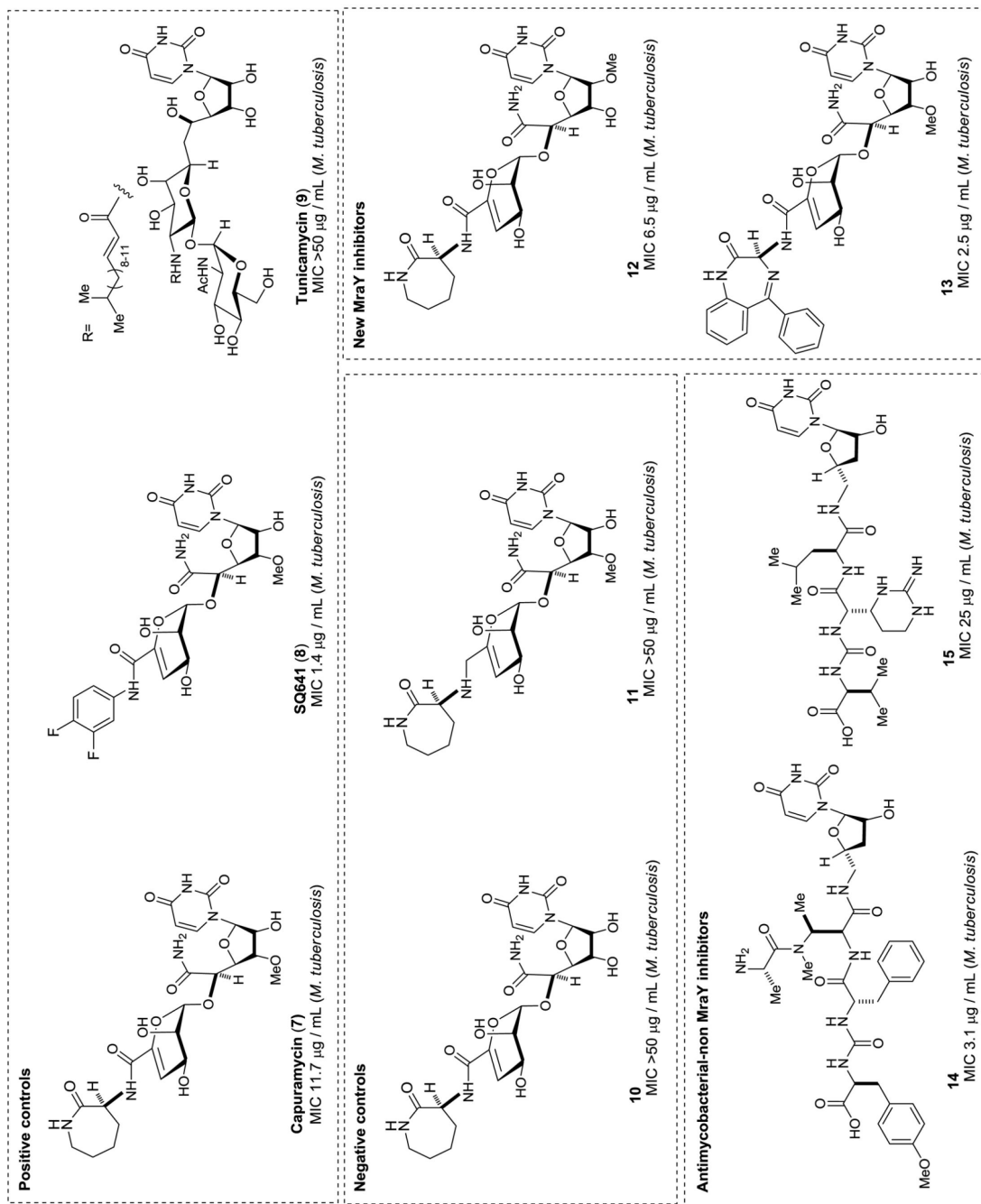


entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
8	13	-	40	95	100	-	0.0950 $\pm$ 0.00395

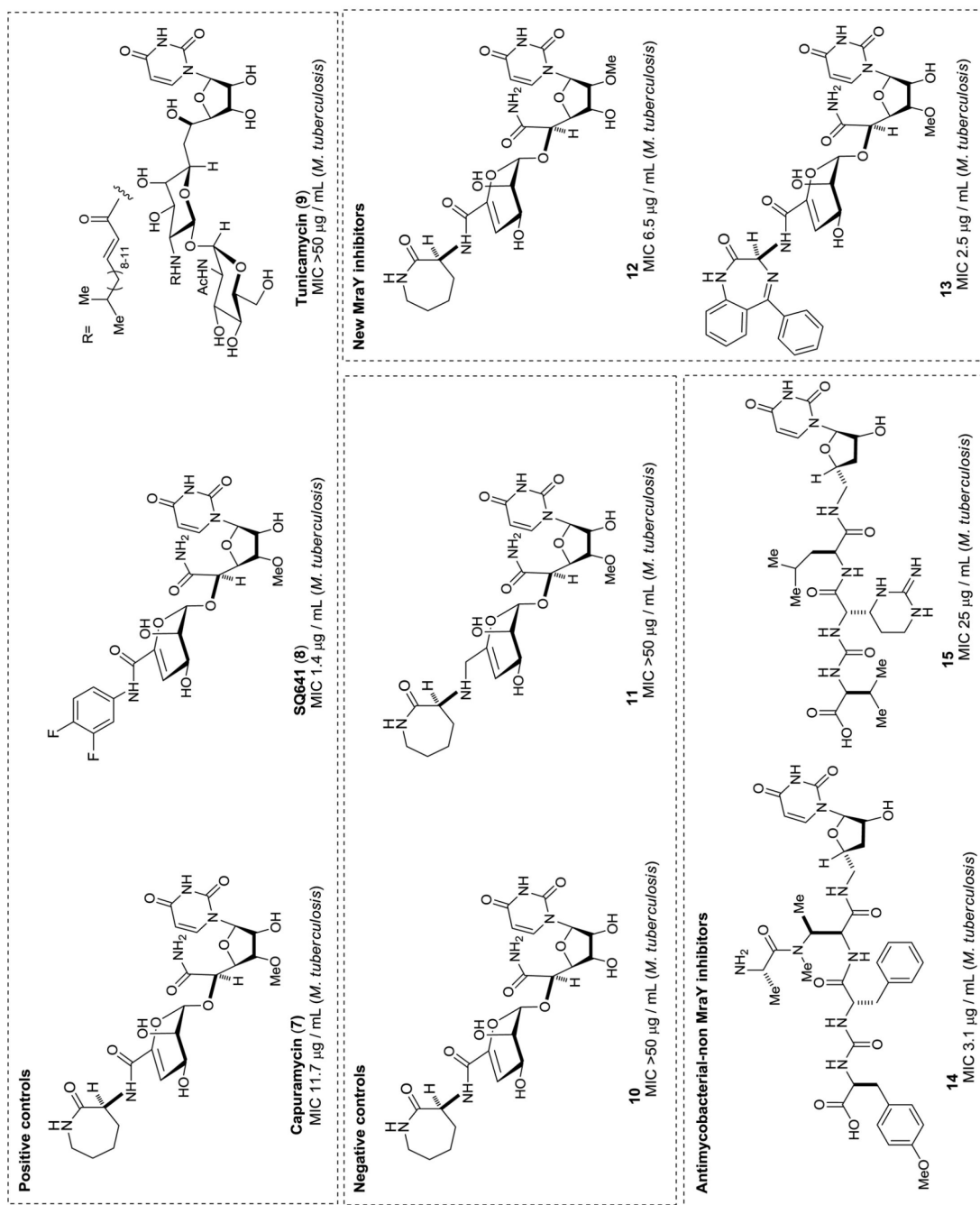




entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
9	14	-	0	0	0	0	ND



entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
10	15	-	0	0	0	0	ND



entry	Compound	MurX inhibition (%) <sup>a</sup>					IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>
		0.01 $\mu\text{M}$	0.1 $\mu\text{M}$	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$	
11	DMSO	0	0	0	0	0	ND

<sup>a</sup> 1) Reaction conditions: Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2** (75 μM; 3.75 μL), MgCl<sub>2</sub> (0.5 M; 10 μL); KCl (2 M, 10 μL), Triton X100 (0.1%; 11.25 μL), Tris-buffer (pH = 8; 50 mM, 2.5 μL), neryl phosphate **6** (10 mM, 45 μL), inhibitor molecule (0-100 μM in DMSO (2.5 μL)), P-60 (15 μL), 26 °C, 3h.; The *K<sub>m</sub>* 18.29 μM (Park's nucleotide **2**).

<sup>b</sup> The IC<sub>50</sub> values were obtained three times and the standard error of the mean was calculated.

<sup>c</sup> The IC<sub>50</sub> values in parentheses were reported in the literatures (see, references 18, 19, 24, 25, 45) and/or were obtained *via* the assay conditions summarized in Table 1.; Reaction conditions: Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea **2** (75 μM; 3.75 μL), MgCl<sub>2</sub> (0.5 M; 10 μL); KCl (2 M, 10 μL), Triton X100 (0.1%; 11.25 μL), Tris-buffer (pH = 8; 50 mM, 5 μL), decaprenyl phosphate (10 mM, 2 equivalents against **2**), inhibitor molecule (0-100 μM in DMSO (2.5 μL)), P-60 (15 μL), 26 °C, 3h.; The *K<sub>m</sub>* 18.05 μM (Park's nucleotide **2**).