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Chemoenzymatic syntheses of water-soluble lipid I fluorescent probes

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This Letter is dedicated to the memory of Professor Harry H. Wasserman, an inspirational scientist

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ABSTRACT

Peptidoglycan (PG) is unique to bacteria, and thus, the enzymes responsible for its biosynthesis are promising antibacterial drug targets. The membrane-embedded enzymes in PG remain significant challenges in studying their mechanisms due to the fact that preparations of suitable enzymatic substrates require time-consuming biological transformations or chemical synthesis. Lipid I (MurNac(pentapeptide)-pyrophosphoryl prenol) is an important PG biosynthesis intermediate to study the central enzymes, translocase I (MraY/MurX) and MurG. Lipid I isolated from nature contains the C₅₀- or C₅₅-prenyl unit that shows extremely poor water-solubility that renders studies of translocase I and MurG enzymes difficult. We have studied biological transformation of water soluble lipid I fluorescent probes using bacterial membrane fractions and purified MraY enzymes. In our investigation of the minimum structural requirements of the prenyl phosphates in MraY-catalyzed lipid I synthesis, we found that (2*Z*,6*E*)-farnesyl phosphate (C₁₅-phosphate) can be recognized by *Escherichia coli* MraY to generate the water-soluble lipid I fluorescent probe in high-yields. Under the optimized conditions, the same reaction was performed by using the purified MraY from *Hydrogenivirga* spp. to afford the lipid I analog with high-yields in a short reaction time.

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Since peptidoglycan (PG) is an essential bacterial cell-wall polymer, the machinery for PG biosynthesis provides a unique and selective target for antibiotic action.^{1–3} 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.^{4,5} We have been studying bacterial phosphotransferases and glycosyltransferases aiming at developing new antibacterial agents for multi-drug resistant (MDR) pathogens.^{5–13} Especially, the membrane-associated proteins translocase I (MraY/MurX) and MurG, which catalyze the transformations from Park's nucleotide (UDP-MurNac-pentapeptide) to lipid I (MurNac(pentapeptide)-pyrophosphoryl prenol) and from lipid I to lipid II (GlcNac-MurNac(pentapeptide)-pyrophosphoryl prenol), are our research interests (Fig. 1). Syntheses of lipid I and lipid II have been achieved by total chemical synthetic or chemoenzymatic approaches; however, these processes require sophisticated synthetic techniques and skills in purifying amphiphilic molecules.^{6,14–19} In addition, the cost of acquisition of enough polyprenyl phosphate is very high. Recently, we reported biosynthesis of a

water-soluble lipid I-nyl (C₁₀) analog **7** from Park's nucleotide-N⁶-dansylthiourea **1** (Figs. 2 and 3) and neryl phosphate using *Mycobacterium smegmatis* P-60 (MraY-containing membrane fraction).⁵ A water-soluble lipid I generated in the reaction could be quantitated conveniently via reverse-phase HPLC without tedious extraction procedures. These synthetic protocols could be applied to a development of robust MraY/MurX assay for identifying novel antibacterial agents.⁵ Investigation of versatile MraY/MurG enzyme substrate mimics that can efficiently be transformed to lipid I and lipid II fluorescent derivatives requires further structural studies of Park's nucleotide and lipid I. In the present Letter, we report a new Park's nucleotide fluorescent probe **4** that can be recognized by MraY and MurG from a wide range of bacteria and the efficient biosynthesis of a water soluble lipid I fluorescent probe **10** with *Escherichia coli* MraY and a purified MraY.

We realized that MurX- or MraY-containing membrane fractions (P-60)²⁰ obtained from *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, and *E. coli* could recognize Park's nucleotide fluorescent probes (**1–3**, Fig. 2) and they could be converted to the corresponding lipid I analogs in 15–25% yields when 2–10 equiv of undecaprenyl phosphate was utilized under optimized conditions.^{21–25} On the other hand, Park's nucleotide fluorescent probes (**1–3**) were not effective in the biosynthesis of

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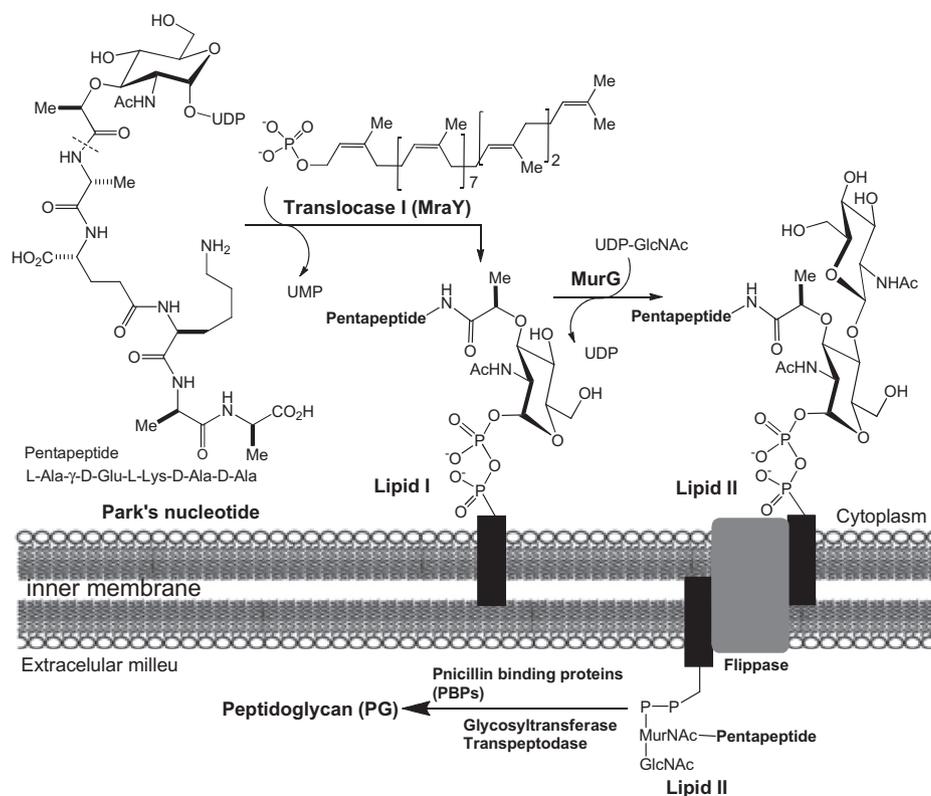


Figure 1. Biosynthesis of lipid II from Park's nucleotide in *E. coli*.

lipid II analogs using P-60 in the presence of UDP-GlcNAc. These results indicate that the binding affinity of lipid I, whose lysine residue was modified directly with commercially available fluorophores, with MurG is markedly decreased. On the other hand, Park's nucleotide-*N*⁶-C6-dansyl **4** was converted to the corresponding lipid I and lipid II in 65–70% yield using *E. coli* P-60 in the absence or presence of UDP-GlcNAc (Fig. 2). It is interesting to note that a nitrobenzoxadiazole (NBD) linked *N*-glycolyl Park's nucleotide was applied to the biosynthesis of lipid II, but the desired lipid II derivative was isolated in a trace amount when *E. coli* membrane fraction was used.²⁶ Thus, we could identify a linker, 6-aminohexan-1-ol that can effectively conjugate the fluorophore with Park's nucleotides without loss of the binding affinities with MraY and MurG. Because Park's nucleotide-*N*⁶-C6-dansyl **4** could be converted to undecaprenyl lipid II via P-60 derived from *M. tuberculosis*, *M. smegmatis*, *S. aureus*, and *E. coli*, it was concluded that **4** is a versatile Park's nucleotide probe to study functions and mechanisms of MraY/MurX and MurG. In order to confirm the efficiency of **4** in the biosynthesis of lipid I-fluorescent probes, a series of experiments were performed. In biosynthesis of neryl-lipid I analog with Park's nucleotide-*N*⁶-dansylthiourea **1**, the reaction with *M. smegmatis* P-60 furnished the neryl-lipid I analog in >50% yield within 2 h, whereas, the same reaction with *E. coli* P-60 did not provide the desired product with <20% (a vs b in Fig. 3). Park's nucleotide-*N*⁶-C6-dansyl **4** has a significant advantage in biosyntheses of lipid I analogs; the corresponding neryl-lipid I derivative **9** could be synthesized with >60% and 80% yields in 3 h using *E. coli* and *M. smegmatis* P-60, respectively (c vs d in Fig. 3). Upon further investigation of minimum structure requirement of the prenyl phosphate in the MraY-catalyzed lipid I analog synthesis with **4**, it was found that (2*Z*,6*E*)-farnesyl phosphate (C₁₅-phosphate) is a better mimic for prenyl phosphate than neryl phosphate (C₁₀-phosphate); in the product/time course experiments, no

obvious difference in reaction rate was observed between *E. coli* and *M. smegmatis* P-60 catalyzed lipid I analog syntheses (e vs f in Fig. 3).

Comparison of kinetic parameters of *E. coli* P-60-catalyzed lipid I-neryl and -farnesyl syntheses with **4** clearly supported the observed reaction rates (c vs f in Fig. 3); *E. coli* MraY has over 5 times higher *K*_m value for neryl phosphate than that for farnesyl phosphate (5.66 × 10³ μM for neryl-P and 9.80 × 10² μM for farnesyl-P). The *V*_{max} for farnesyl-lipid I-*N*⁶-C6-dansyl **10** synthesis by *E. coli* MraY was determined to be 7.96 × 10⁻⁷ μM/s through the Michaelis–Menten plot, whereas 1.23 × 10⁻⁶ μM/s for neryl-lipid I-*N*⁶-C6-dansyl **9**.²⁷ Significant difference in MraY-catalyzed farnesyl-lipid I analog synthesis compared to the neryl-lipid I version can be attributed to the fact that more hydrophobic characteristics of the farnesyl group increases the affinity with the hydrophobic catalytic site of MraY. However, it is worth mentioning that farnesyl phosphate applied in these experiments has 2*Z*,6*E*-configuration, albeit, the natural substrates has 2*Z*,6*Z*-isoprenyl units (Fig. 1).

(2*Z*,6*E*)-Farnesyl phosphate was synthesized from farnesol (**11**) in three steps in >38% overall yield,²⁸ thus, unlike undecaprenyl phosphate, a sufficient amount of the water-soluble counterpart is readily available. Park's nucleotides can be relatively easily synthesized via total chemical synthesis or enzymatic processes using crude MurA–F from UDP-GlcNAc.^{6,9} As illustrated in Scheme 1, the fluorescent linker **14** was synthesized in two steps in >90% overall yield;²⁹ **14** could be utilized for conjugation with Park's nucleotides without purification. Conjugation between Park's nucleotide and **14** was accomplished with ¹Pr₂NEt in water-containing solvents (see, Supporting information).³⁰ Park's nucleotide-*N*⁶-C6-dansyl **4** could be stored in DMSO or in pH 8 Tris-buffer at –20 °C for over three months without detectable degradation. Thus, practical and convenient enzymatic substrates were identified to study MraY and MurG enzymes.

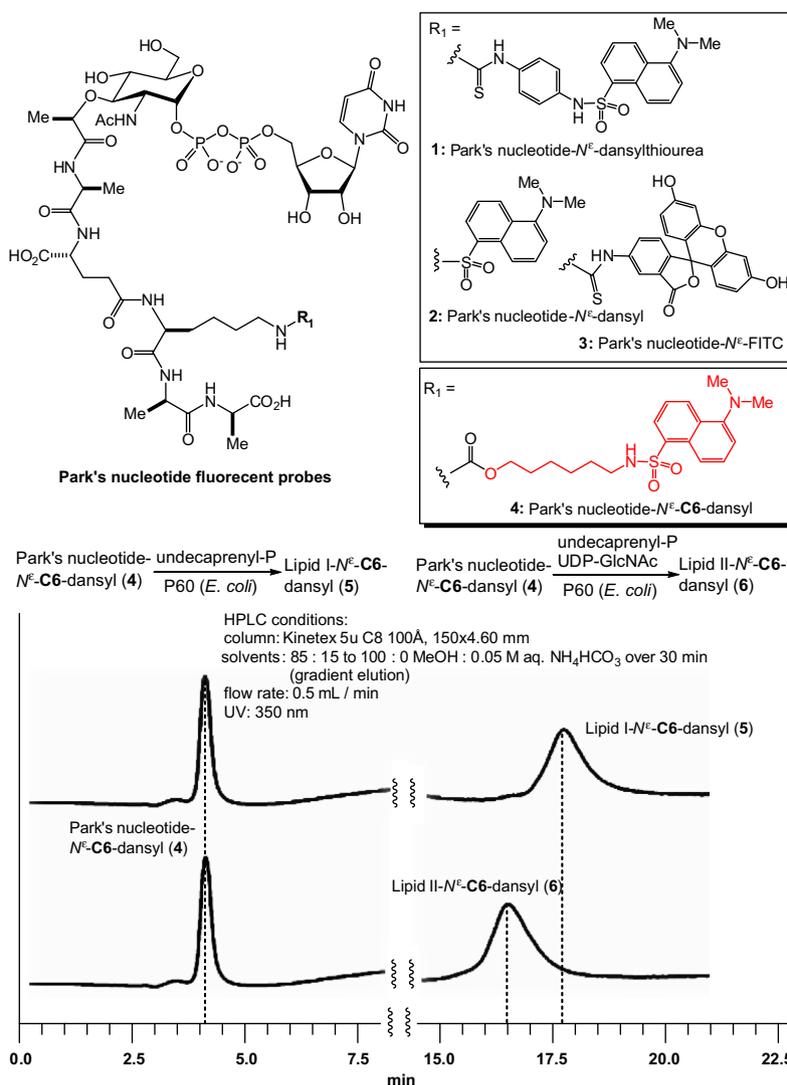


Figure 2. Identification of a fluorescent probe for MraY/MurG-catalyzed biotransformations.

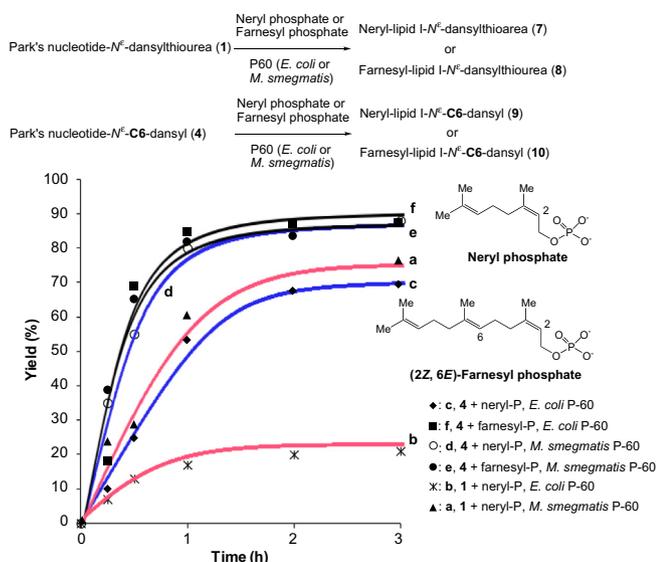
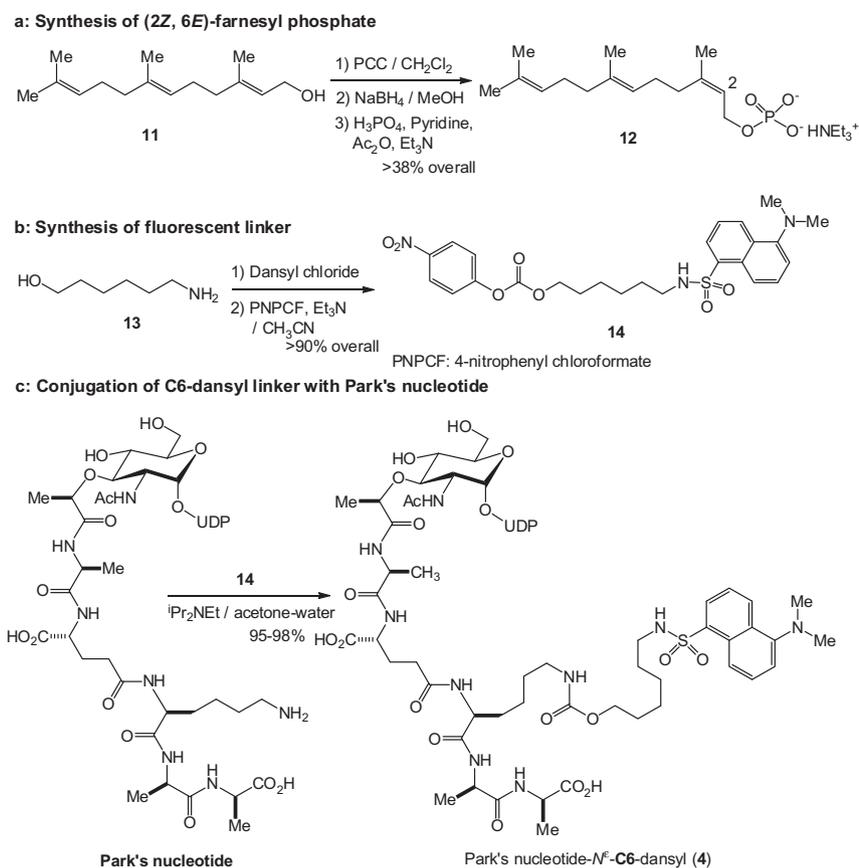


Figure 3. Biosyntheses of lipid I analogs via P-60 membrane fractions.

The MraY-catalyzed transformation of lipid I from Park's nucleotide is believed to be a reversible process.³¹ Therefore, it is difficult to achieve a complete conversion with natural enzyme substrates. On the contrary, under optimized conditions synthesis of farnesyl-lipid I **10** with Park's nucleotide-*N*⁶-C6-dansyl **4** and *E. coli* P-60 can be achieved in >80% yield within 1 h when 60 equiv of farnesyl phosphate **12** was used (Fig. 4). Even using 3 equiv of **12**, a useful level of conversion was achieved for studying functions of MraY.

For structural and functional studies, recombinant MraY enzymes from thermophilic eubacteria, such as *Aquifex* spp. or *Hydrogenobacter* spp. have been expressed and purified by several research groups.³² In our attempt at development of a rapid and practical preparation of lipid I fluorescent probes, the purified MraY (40.1 kDa) from *Hydrogenovirga* spp. 128-5-R1-1 was applied for the synthesis of farnesyl-lipid I-*N*⁶-C6-dansyl **10**.³³ When HyMraY was applied to the transformation from Park's nucleotide-*N*⁶-dansylthiourea **1** to neryl-lipid I-*N*⁶-dansylthiourea **7** with neryl phosphate (60 equiv), denaturing of the purified MraY was observed during long reaction time required for neryl-lipid I synthesis; the reaction was terminated in 40–60% conversion within 1 h, whereas, the same reaction with *M. smegmatis* P-60 furnished



Scheme 1. Syntheses of *MraY* enzyme substrates for biotransformation from **4** to **10**.

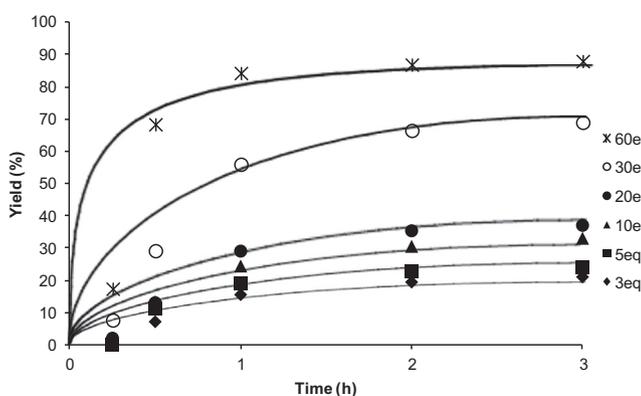


Figure 4. Effect of concentrations of (2Z,6E)-farnesyl phosphate on biosynthesis of farnesyl-lipid I-*N*⁶-C6-dansyl **10**.

the desired product **7** in near quantitative yields after 12 h (in 0.075 mM solution for Park's nucleotide).⁵ Using membrane fractions (e.g., P-60) is a convenient approach for pilot scale syntheses and especially for biological assays. However, the reactions using P-60 membrane fractions are impractical when one aims to synthesize over-milligram quantities of the substrates due to a requirement of large volume of P-60 and a phase-transfer catalyst (detergent). In contrast, *HyMraY*-catalyzed lipid I synthesis with Park's nucleotide-*N*⁶-C6-dansyl **4** and farnesyl phosphate **12** (30 equiv) could be accomplished within 1 h to afford **10** in quantitative yields. Notably, this reaction could readily be scaled-up for the synthesis of a few milligram of **10** with *HyMraY* (20 μg) in 0.3 mM concentrations (for Park's nucleotide) (Fig. 5). The reaction

mixture was lyophilized and filtered through a membrane filter to afford the crude product, which was purified by reverse-phase HPLC (solvent: CH₃CN/0.05 M aq. NH₄HCO₃ = 25:75), yielding **10** in 80–90% yield.³⁴ All physical data including the retention time (in HPLC) of farnesyl-lipid I-*N*⁶-C6-dansyl **10** synthesized in Figure 5 agreed with **10** synthesized via a total chemical synthesis.³⁵

In summary, we have demonstrated efficient biosynthesis of a water-soluble lipid I fluorescent probe with a convenient membrane fraction (P-60) prepared from *E. coli*.³⁶ In previous works, biosynthesis of lipid I analogs with Park's nucleotide-*N*⁶-dansylthiourea **1** was demonstrated via P-60 from *Mycobacterium* spp; the affinity of **1** with the other *MraY* enzymes are weaker than that from *Mycobacterium* spp.⁵ A new probe, Park's nucleotide-*N*⁶-C6-dansyl **4** exhibited sufficient affinity with translocase I enzymes of a wide range of bacterial species.³⁷ (2Z,6E)-Farnesyl phosphate **12** was identified as a water soluble counterpart of undecaprenyl phosphate for *MraY* enzymes. The combination of the *MraY* enzyme substrate mimics, **4** and **12** enabled the syntheses of lipid I analogs with a purified *MraY* at high concentrations; as demonstrated in Figure 5 a few milligram quantities of farnesyl-lipid I-*N*⁶-C6-dansyl **10** could be synthesized with *HyMraY* (~20 μg) within 1 h. We have accomplished the synthesis of over 300 mg quantities of Park's nucleotide.⁹ Therefore, lipid I and lipid II fluorescent probes can be synthesized chemoenzymatically according to the procedures illustrated in Scheme 1 and Figure 3. Lipid I-*N*⁶-C6-dansyl **5** was recognized by MurG to form the corresponding lipid II **6**. A water-soluble lipid I-*N*⁶-C6-fluorescent probe will be a valuable asset to study functions of MurG and the other enzymes associated with MurG.^{38–40} New lipid I fluorescent probes and their kinetic parameters for MurG will be disclosed elsewhere.

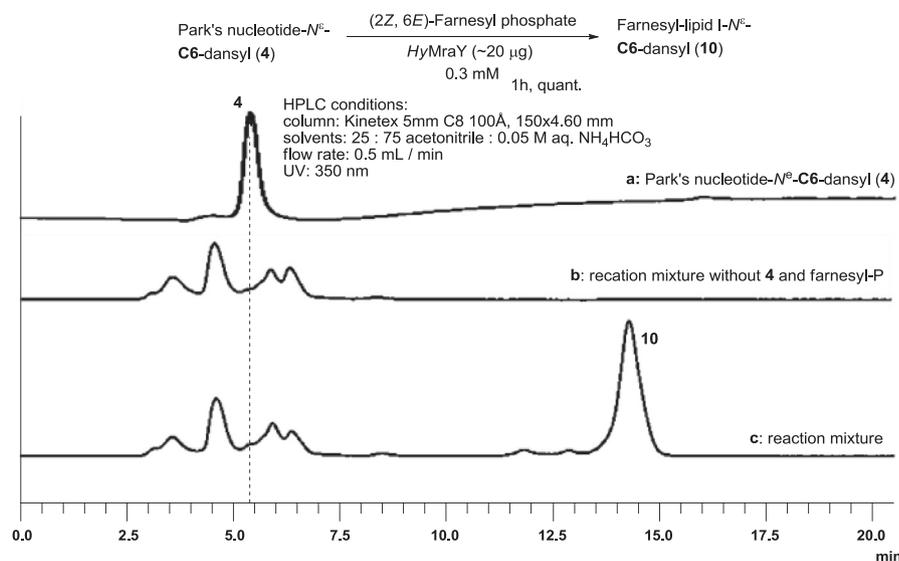


Figure 5. HyMraY-catalyzed transformation from **4** to **10**.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tetlet.2015.01.044>.

References and notes

- Van Heijenoort, J. *Nat. Prod. Rep.* **2001**, *18*, 503–519.
- Higashi, Y.; Strominger, J. L.; Sweeley, C. C. *J. Biol. Chem.* **1970**, *245*, 3697–3702.
- Parr, T. R.; Saier, M. H. *Res. Microbiol.* **1992**, *143*, 443–447.
- Wright, G. D. *Science* **2007**, *315*, 1373–1374.
- Siricilla, S.; Mitachi, K.; Skorupinska-Tudek, K.; Swiezewska, E.; Kurosu, M. *Anal. Biochem.* **2014**, *461*, 36–45.
- Kurosu, M.; Mahapatra, S.; Narayanasamy, P.; Crick, D. C. *Tetrahedron Lett.* **2007**, *48*, 799–803.
- Kurosu, M.; Li, K.; Crick, D. C. *Org. Lett.* **2009**, *11*, 2393–2397.
- Kurosu, M.; Narayanasamy, P.; Crick, D. C. *Heterocycles* **2007**, *72*, 339–352.
- Li, K.; Kurosu, M. *Heterocycles* **2008**, *76*, 455–469.
- Aleiwi, B. A.; Schneider, C. M.; Kurosu, M. *J. Org. Chem.* **2012**, *77*, 3859–3867.
- Wang, Y.; Siricilla, S.; Aleiwi, B. A.; Kurosu, M. *Chem. Eur. J.* **2013**, *19*, 13847–13858.
- Siricilla, S.; Mitachi, K.; Wan, B.; Franzblau, S. G.; Kurosu, M. *J. Antibiot.* **2014**, *58*, 6413–6423.
- Tanaka, S.; Clemons, W. M. *Mol. Microbiol.* **2012**, *85*, 975–985.
- Hitchcock, S. A.; Eid, C. N.; Aikins, J. A.; Zia-Ebrahimi, M.; Blaszcak, L. C. *J. Am. Chem. Soc.* **1998**, *120*, 1916–1917.
- Van Nieuwenhze, M. S.; Mauldin, S. C.; Zia-Ebrahimi, M.; Aikins, J. A.; Blaszcak, L. C. *J. Am. Chem. Soc.* **2001**, *123*, 6983–6988.
- Schwartz, B.; Markwalder, J. A.; Wang, Y. *J. Am. Chem. Soc.* **2001**, *123*, 11638–11643.
- Saha, S. L.; Van Nieuwenhze, M. S.; Hornback, W. J.; Aikins, J. A.; Blaszcak, L. C. *Org. Lett.* **2001**, *3*, 3575–3577.
- Lebar, M. D.; Lupoli, T. J.; Tsukamoto, H.; May, J. M.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2013**, *135*, 4632–4635.
- Shih, H. W.; Chen, K. T.; Cheng, T. J. R.; Wong, C. H.; Cheng, W. C. *Org. Lett.* **2011**, *13*, 4600–4603.
- Rezwan, M.; Laneelle, M. A.; Sander, P.; Daffe, M. *J. Microbiol. Methods* **2007**, *68*, 32–39.
- Typical procedure using P-60 membrane fraction: Tris-buffer (pH = 8.0; 50 mM, 38.7 μ L), Triton X 100 (0.5%; 11.2 μ L), MgCl₂ (0.5 M; 10 μ L), KCl (2 M, 10 μ L), prenyl phosphate (20 mM, 2–60 equiv), and Park's nucleotide (2 mM; 3.8 μ L) were placed in a 1.5 mL Eppendorf tube. To a reaction mixture, P-60 membrane fractions (1 mg/ μ L, 15 μ L) were added. The reaction mixture was incubated for 1–15 h at room temperature and quenched with CHCl₃ (200 μ L). Two phases were centrifuged at 25,000g for 10 min. The upper aqueous phase was collected. 10 μ L of the aqueous phase was injected into the reverse-phase HPLC (CH₂CN/0.05 M NH₄HCO₃ = 25:75, UV: 350 nm, flow rate: 0.5 mL/min, Column: Kinetex 5u C8 100 Å, 150 \times 4.60 mm) and the area of the peak for the lipid I derivative was quantified to obtain the product yield.
- Reddy, S. G.; Waddell, S. T.; Kuo, D. W.; Wong, K. K.; Pompliano, D. L. *J. Am. Chem. Soc.* **1999**, *121*, 1175–1178.
- Weppner, W. A.; Neuhaus, F. C. *J. Biol. Chem.* **1977**, *77*, 2296–2303.
- Sadamoto, R.; Niikura, K.; Sears, P. S.; Liu, H.; Wong, C.-H.; Suksomcheep, A.; Tomita, F.; Monde, K.; Nishimura, S. *J. Am. Chem. Soc.* **2002**, *124*, 9018–9019.
- Sadamoto, R.; Niikura, K.; Ueda, T.; Monde, K.; Fukuhara, N.; Nishimura, S. *J. Am. Chem. Soc.* **2004**, *126*, 3755–3761.
- Chen, K. T.; Kuan, Y. C.; Fu, W. C.; Liang, P. H.; Cheng, T. J. R.; Wong, C. H.; Cheng, W. C. *Chem. Eur. J.* **2013**, *19*, 834–838.
- Ma, Y.; Münch, D.; Schneider, T.; Sahl, H. G.; Bouhss, A.; Ghoshdastider, U.; Wang, J.; Dötsch, V.; Wang, X.; Bernhard, F. *J. Biol. Chem.* **2011**, *286*, 38844–38853.
- Data for (2Z,6E)-farnesyl phosphate **12**: ¹H NMR (400 MHz, deuterium oxide) δ 5.40 (t, *J* = 7.3 Hz, 1H), 5.20–5.12 (m, 2H), 4.35 (t, *J* = 7.0 Hz, 2H), 2.15–2.00 (m, 8H), 1.73 (s, 3H), 1.66 (s, 3H), 1.59 (s, 6H); LRMS (EI) calcd for C₁₅H₂₈O₄P [M+H]⁺: 303.17, found: 303.19.
- Data for the linker **14**: ¹H NMR (400 MHz, chloroform-*d*) δ 8.61 (d, *J* = 8.5 Hz, 1H), 8.33 (d, *J* = 7.4 Hz, 1H), 8.30–8.24 (m, 1H), 8.27 (d, *J* = 9.2 Hz, 2H), 7.57 (q, *J* = 7.9 Hz, 2H), 7.37 (d, *J* = 9.2 Hz, 2H), 7.25 (br s, 1H), 4.74 (t, *J* = 6.2 Hz, 1H), 4.18 (t, *J* = 6.6 Hz, 2H), 2.95 (s, 6H), 2.97–2.85 (m, 2H), 1.65–1.56 (m, 2H), 1.47–1.38 (m, 2H), 1.29–1.21 (m, 4H); LRMS (EI) calcd for C₂₅H₃₀N₃O₃S [M+H]⁺: 516.18, found: 516.26.
- Data for Park's nucleotide-*N*⁶-C6-dansyl (**4**): ¹H NMR (400 MHz, deuterium oxide) δ 8.46 (d, *J* = 8.6 Hz, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 8.23 (d, *J* = 7.3 Hz, 1H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.66 (q, *J* = 7.4 Hz, 2H), 7.38 (d, *J* = 7.6 Hz, 1H), 5.91 (d, *J* = 3.7 Hz, 1H), 5.89 (d, *J* = 7.9 Hz, 1H), 5.41 (dd, *J* = 7.3, 3.2 Hz, 1H), 4.32–4.26 (m, 2H), 4.25–4.02 (m, 10H), 3.90 (dt, *J* = 9.7, 3.0 Hz, 1H), 3.82–3.70 (m, 3H), 3.66–3.54 (m, 3H), 3.04 (t, *J* = 6.3 Hz, 2H), 2.92 (t, *J* = 6.5 Hz, 2H), 2.84 (s, 6H), 2.26–2.19 (m, 2H), 2.13–2.02 (m, 1H), 1.95 (s, 3H), 1.87–1.78 (m, 1H), 1.74–1.62 (m, 2H), 1.47–1.32 (m, 4H), 1.37 (d, *J* = 7.2 Hz, 3H), 1.34 (d, *J* = 6.7 Hz, 3H), 1.29 (d, *J* = 7.7 Hz, 3H), 1.27 (d, *J* = 7.6 Hz, 3H), 1.13–1.06 (m, 2H), 1.04–0.97 (m, 2H), 0.85–0.68 (m, 4H); LRMS (EI) calcd for C₅₉H₉₀N₁₁O₃₀P₂S [M+H]⁺: 1526.51, found: 1526.40.
- Stachyra, T.; Dini, C.; Ferrari, P.; Bouhss, A.; van Heijenoort, J.; Mengin-Lecreulx, D.; Blanot, J. D.; Biton, D.; Le Beller, D. *Antimicrob. Agents Chemother.* **2004**, *48*, 897–902.
- Chung, B. C.; Zhao, J.; Gillespie, R. A.; Kwon, D.-Y.; Guan, Z.; Hong, J.; Zhou, P.; Lee, S.-Y. *Science* **2013**, *341*, 1012–1016.
- MraY, a pET22b vector containing an N-terminal 6 \times Histagged HyMraY was used to transform Nico(pLemo) cells for expression.
- Synthesis of lipid I analog with HyMraY: Tris-buffer (pH = 8.0; 50 mM, 1.7 μ L), Triton X 100 (22%; 0.2 μ L), MgCl₂ (2.5 M; 2 μ L), KCl (4 M, 5 μ L), (2Z,6E)-farnesyl phosphate (20 mM, 11.3 μ L), and Park's nucleotide-*N*⁶-C6-dansyl (2 mM; 3.8 μ L) were placed in a 1.5 mL Eppendorf tube. To a stirred reaction mixture, HyMraY (~0.1 mg/mL, 6 μ L) was added (total volume of reaction mixture: 30 μ L). The reaction mixture was incubated for 1 h at room

temperature. The reaction mixture was lyophilized and filtered through a membrane filter. The crude product was purified by reverse-phase HPLC (CH₃CN/0.05 M ammonium bicarbonate = 25:75). This reaction was scaled-up by using Park's nucleotide-*N*⁶-C6-dansyl (1.5 mg) and (2*Z*,6*E*)-farnesyl phosphate (9 mg) in total volume of 1.5 mL.

35. Mitachi, K.; Mohan, P.; Siricilla, S.; Kurosu, M. *Chem. Eur. J.* **2014**, *20*, 4554–4558.
36. Water-solubility of farnesyl-lipid I-*N*⁶-C6-dansyl **10** is over 3.1 mg/mL and **10** could not be extracted with *n*-butanol.

37. The FITC conjugate, Park's nucleotide-*N*⁶-C6-FITC was equally effective in the syntheses of lipid I and lipid II analogs.
38. Pasquina, L. W.; Santa Maria, J. P. S.; Walker, S. *Curr. Opin. Microbiol.* **2013**, *16*, 531–537.
39. Helm, J. S.; Hu, Y.; Chen, L.; Gross, B.; Walker, S. *J. Am. Chem. Soc.* **2003**, *125*, 11168–11169.
40. Kang, C.-M.; Nyayapathy, S.; Lee, J.-Y.; Suh, J. W.; Husson, R. N. *Microbiology* **2008**, *154*, 725–735.