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Rapid Preparation of Mycobacterium *N*-Glycolyl Lipid I and Lipid II Derivatives: A Biocatalytic Approach

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Tuberculosis (TB), an infectious disease induced by *Mycobacterium tuberculosis* (*Mtb*), is a leading cause of death worldwide.[1] The increasing prevalence of drug resistant TB strains, including multidrug-resistant TB (MDR-TB), and extensively drug-resistant TB (XDR-TB), is of growing concern. New, more effective antibiotics are urgently required.[2] The mycobacterial cell wall is essential for viability, and therefore the enzymes responsible for its biosynthesis are possible antibiotic targets. For example, mycobacterial penicillin-binding protein (PBP), PonA, located on the external surface of mycobacterial membranes, possesses two catalytic domains for transpeptidase and transglycosylase activities and plays an important role in the last two steps (transglycosylation and transpetidation) of mycobacterial cell wall constructions.[3] The transpeptidase responsible for the cross-linking of peptidoglycans is a known target but the serious drug resistance has been reported.[4] In contrast, no antibiotics have been developed to target the transglycosylase (TGase).[5]

PonA is relatively easy to access, essential for mycobacterial viability, without a eukaryotic counterpart, and hence an attractive target for antibiotic discovery and development.[6] During transglycosylation, the sugar moiety from the activated polymeric peptidoglycan (a glycosyl donor) is linked to the specific hydroxyl group (4-OH) of *N*-glycolyl Lipid II (a glycosyl acceptor), and a decaprenyl pyrophosphate is released (Figure 1). Structurally, *N*-glycolyl Lipid II consists of the disaccharide of *N*-acetylglucosamine (GlcNAc) and *N*-glycolyl muramic acid (MurNGlyc), pyrophosphate, decaprenol lipid tail, and a pentapeptide moiety (L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine). Another key component, *Mtb* *N*-glycolyl Lipid I, consisting of *N*-glycolyl muramic acid, decaprenol lipid tail, and the pentapeptide moiety, is a biosynthetic precursor of *N*-glycolyl Lipid II. MurNGlyc is only observed in mycobacterial cell walls, and is therefore considered a potential biomarker.[7] The *N*-glycolyl groups in peptidoglycan chains may play an critical role for the resistance to lysozyme and for the innate immune response during a mycobacterial infection.[8]

During the late stages of peptidoglycan biosynthesis, the enzyme *Mtb* MraY catalyzes the transfer of the sugar moiety from *Mtb* *N*-glycolyl Park's nucleotide (UDP-*N*-glycolyl-muramyl-L-alanyl-D-glutamyl-*meso*- diaminopimelyl-D-alanyl-D-alanine) to the decaprenyl phosphate (C50P), to give *N*-glycolyl Lipid I. Subsequently, conjugation of *Mtb* *N*-glycolyl Lipid I with UDP-GlcNAc is catalyzed by *Mtb* MurG to produce *Mtb* *N*-glycolyl Lipid II (Figure 1). It is noteworthy that both *Mtb* MraY and *Mtb* MurG are membrane-associated proteins in mycobacterium.

However, mechanistic and inhibitor studies for late-stage peptidoglycan biosynthesis have been hampered by the difficulty in acquiring pure samples of membrane-associated materials such as C50P, *N*-glycolyl Lipid I, and *N*-glycolyl Lipid II. Direct isolation of these materials from mycobacterium is difficult due to their low natural abundance and structural complexity.[9] Recently, a multi-step chemical synthesis of structurally modified *N*-glycolyl Park's nucleotide[10] and *N*-glycolyl Lipid II,[11] were reported. A similar synthesis of *N*-glycolyl Lipid I, however, remains unknown. In previous work, we demonstrated that the decaprenyl phosphate and *meso*-DAP of the natural *Mtb* *N*-glycolyl Lipid II could be substituted by two accessible materials, undecaprenyl phosphate (C55P) and L-lysine, respectively, and that this synthetic *N*-glycolyl Lipid II is recognized as a PonA substrate.[11]

As part of our ongoing interest in the development of new methods for the preparation of mycobacterial cell wall components, we realized that elaboration of *N*-glycolyl Park's nucleotide to more complex molecules through biocatalytic synthesis might be an attractive alternative to conventional chemical synthesis, the utility of which is limited by tedious chemical transformations such as glycosylation, pyrophosphate formation, and protection/deprotection steps.[12] The use of purified or crude enzymes as biocatalysts for chemical transformations is a promising approach due to their high chemo-, regio- and enantioselectivity, and mild re-

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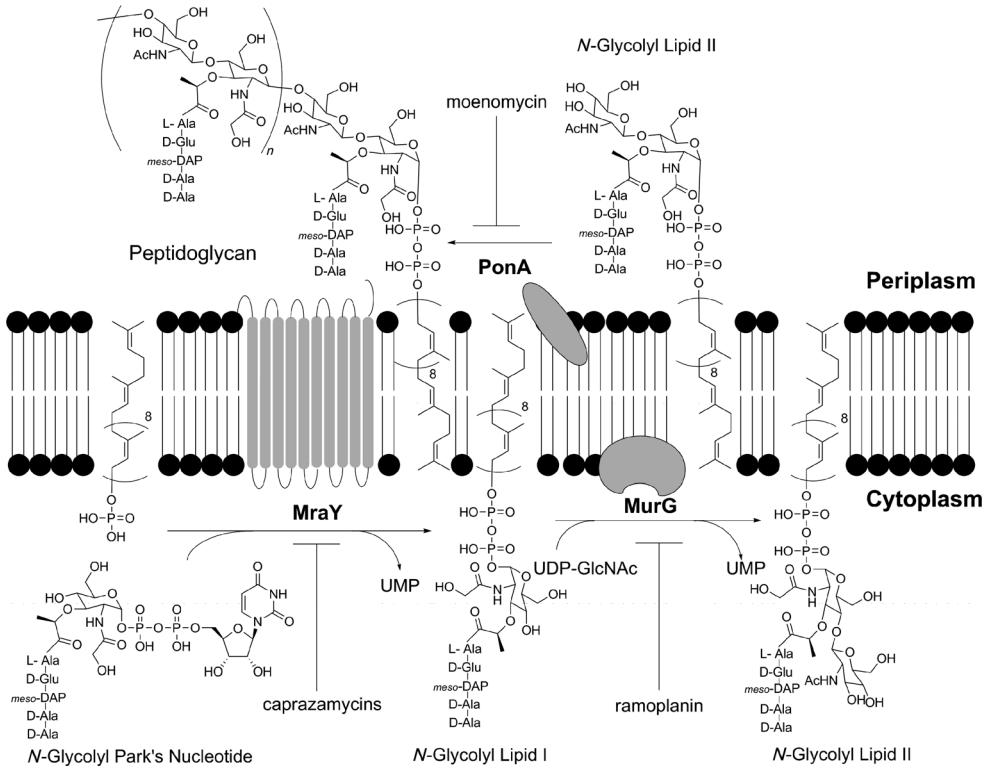


Figure 1. Biosynthesis of mycobacterial peptidoglycan.

action conditions.[13] Several biocatalysts have been extensively studied and commercialized for applications including transesterification, aminolysis, and acidolysis.[14] To the best of our knowledge, preparation of mycobacterial *N*-glycolyl Lipid I, Lipid II, and their analogues through biocatalytic synthesis has not been extensively explored.[15] Herein, we describe the preparation of structurally complex mycobacterial *N*-glycolyl Lipid I and Lipid II-based molecules through biocatalytic synthesis from *N*-glycolyl Park's nucleotide derivatives (Figure 2). The bioactivities of these synthetic molecules towards PonA are also evaluated.

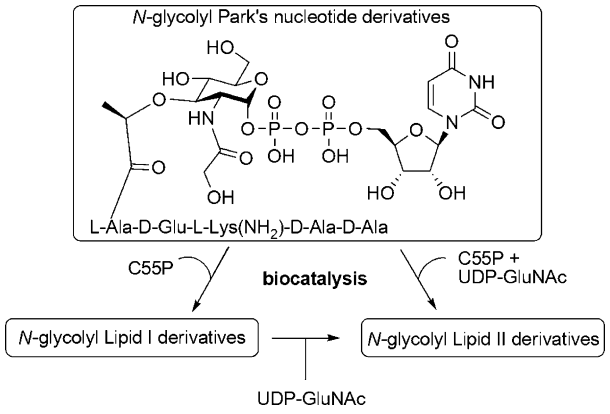
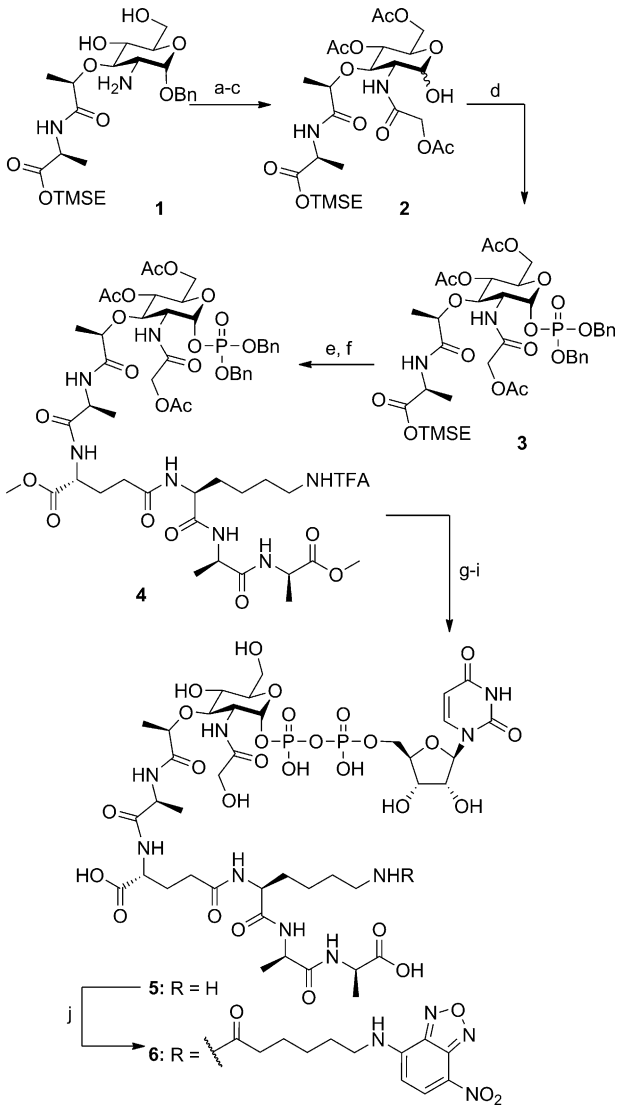


Figure 2. General strategy for the preparation of mycobacterial *N*-glycolyl Lipid I and Lipid II-based molecules.

Our synthesis of *N*-glycolyl Park's nucleotide derivatives started from the protected muramic acid ester **1** (Scheme 1).[11] *N*-Glycolylation of **1** using *N*-succinimidyl acetoxyacetate under basic conditions followed by acetylation of diols at C4 and C6, and O-debenzylation through catalytic hydrogenation gave **2** in 63% overall yield over three steps. Several conditions to accomplish the O-debenzylation were investigated; the use of catalytic $Pd(OH)_2$ in the presence of 0.1% conc. HCl in THF resulted in the best yield (75%). In contrast, when the reactants were dissolved in methanol, or when a small amount of glacial acetic acid was added, no satisfactory results (<40% yield) were obtained (see the Supporting Information, Table S1).

The phosphorylation of **2** was carried out in a phosphitylation/oxidation sequence to deliver phosphoryldiester **3** as a single diastereomer in an excellent yield (90%). The α-configuration of **3** was confirmed by ^{1}H NMR spectroscopy. Selective deprotection of the *O*-trimethylsilylethyl (TMSE) group in **3** by treatment with TBAF in THF, followed by coupling with the tetrapeptide D-Glu(OMe)-L-Lys(TFA)-D-Ala-D-Ala-COOMe, gave **4**. Debenzylation of **4** followed by conjugation with UMP-morpholine-*N,N'*-dicyclohexylcarboxamidine salt and global deprotection under basic conditions gave *N*-glycolyl Park's nucleotide **5** in 39% yield. A fluorescent probe **6** was prepared from **5** by attaching a nitronbenzoxadiazole (NBD) fluorophore at the terminal-NH_2 site of lysine on the peptide side chain.

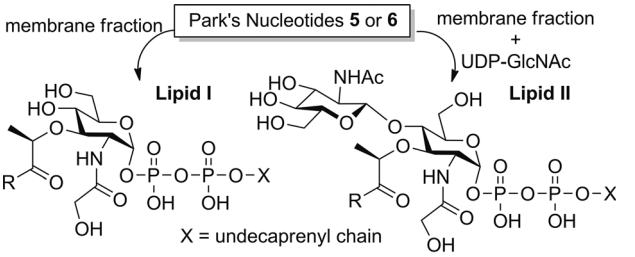
A cell-free membrane fraction from *Mycobacterium smegmatis* was first tested as a biocatalyst. A mixture of the fluo-



Scheme 1. Synthesis of *N*-glycolyl Park's nucleotide and analogues. a) *N*-succinimidyl acetoxyacetate, $NaHCO_3$, 1,4-dioxane, RT, 2 h; b) Ac_2O, pyridine, 2 h, 0 °C to RT; c) $Pd(OH)_2/H_2$, THF/0.1 % conc. HCl, overnight, RT, 65 % (3 steps); d) 1) $iPr_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, RT; 2) *t*BuOOH, −40 °C, 84 %; e) TBAF, THF, RT, 2 h; f) tetrapeptide (D-Glu(OMe)-L-Lys(TFA)-D-Ala-D-Ala(OMe)), PyBOP, DIEA, THF/CH_2Cl_2, RT, 73 % (2 steps); g) $Pd(OH)_2/H_2$, MeOH, RT, 1 h; h) UMP-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt, 1*H*-tetrazole, pyridine, 4 Å molecular sieves, 0 °C to RT, 48 h; i) LiOH, MeOH, RT, 4 h, 39 % (3 steps); j) NBD-X-OSu, $NaHCO_3$, DMF/H_2O, RT, overnight, 90 %.

rescent NBD-labeled Park's nucleotide **6** (2 μmol), UDP-GlcNAc (10 μmol), and C55P (1.5 μmol) in the presence of membrane fraction of *M. smegmatis* was shaken at room temperature. After 30 min, the NBD-labeled *N*-glycolyl Lipid II **9** could be detected by TLC and HPLC analysis. After 1 h, the reaction was quenched by the addition of pyridine acetate buffer (pH 5),[12] though a detectable amount of **6** still remained. Compound **9** was purified by HPLC in 31 % yield based on the initial amount of **6** and 13 % of **6** was recovered (Table 1, entry 1; see the Supporting Information

Table 1. Biocatalytic synthesis of *N*-glycolyl Lipid I and Lipid II.



7: R = L-Ala-D-Glu-L-Lys(NBD)-D-Ala-D-Ala
8: R = L-Ala-D-Glu-L-Lys(NH_2)-D-Ala-D-Ala
9: R = L-Ala-D-Glu-L-Lys(NBD)-D-Ala-D-Ala
10: R = L-Ala-D-Glu-L-Lys(NH_2)-D-Ala-D-Ala

Entry	Substrate [μmol]	Conditions (membrane fraction source/*t* [h])	Product	Yield [%][c]
		Lipid II Synthesis[a]		
1	**6**, 2	*M. smegmatis*[d]/1	**9**	30[e]
2	**6**, 2	*M. smegmatis*/24	**9**	50
3	**6**, 1	*S. aureus*[d]/1	**9**	28
4	**6**, 1	*M. smegmatis*/1	**9**	61
5	**6**, 1	*M. flavus*[d]/1	**9**	72
6	**6**, 1	*E. coli*/1	**9**	trace[f]
7	**5**, 1	*M. flavus*/1	**10**	85
		Lipid I Synthesis[b]		
8	**6**, 1	*M. flavus*/3	**7**	75
9	**5**, 1	*M. flavus*/3	**8**	80

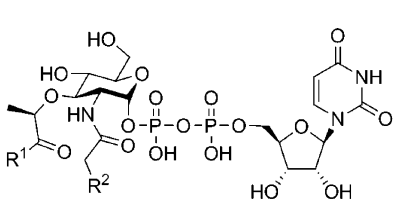
[a] Reaction mixture contains UDP-GlcNAc, C55P, and cell-free membrane fraction. [b] Reaction mixture contains C55P, and cell-free membrane fraction. [c] Isolated product yield after HPLC purification. [d] *M. smegmatis* = *Mycobacterium smegmatis*; *M. flavus* = *Micrococcus flavus*; *S. aureus* = *Staphylococcus aureus* [e] Excess NBD-*N*-glycolyl Park's nucleotide was recovered in 13 %. [f] Only a trace of **9** was observed by using TLC.

Figure S1). This result suggested that the fluorescent probe **6** is indeed a biocatalytic substrate, although the conditions used were not optimized. Extension of the reaction time to 24 h resulted in **9** being isolated in only 50 % yield; some unknown impurities were also observed (Table 1, entry 2). After the initial amount of **6** was adjusted to the final concentration of 1 μmol, the reaction yield of **9** was dramatically improved to 61 % (Table 1, entry 3; see Supporting Information Figure S2). These positive results prompted us to further systematically investigate the reaction conditions (Table 1). Then, other cell-free membrane fractions, including that from *Micrococcus flavus* (Gram-(+)), *S. aureus* (Gram-(+)), and *E. coli* (Gram-(−)) were subsequently evaluated.[16] Several interesting observations should be noted, for example, *S. aureus*, *M. smegmatis* and *M. flavus* membrane fractions catalyzed the formation of NBD-*N*-glycolyl Lipid II in a yield of 28, 61 and 72 %, respectively, but no significant product was observed in the reaction mixture containing *E. coli* membrane fraction (Table 1, entries 4–6). This finding suggests that the catalytic efficiency depends on enzyme capacity. As these membrane fractions are likely to contain membrane-associated TGases, the TGase inhibitor moenomycin was added to the reaction mixture to block transglycosylation and to maximize the yield of Lipid II. Analysis of reaction progress curves did not show a signifi-

cant change, suggesting that either the reaction conditions or the quality of TGase present were unsuitable for transglycosylation (see the Supporting Information, Figure S1).

Use of the cell-free membrane fraction from *M. flavus* as the biocatalyst source for the preparation of Lipid II **9** (Table 1, entry 5) was found to be the most convenient because of its easy accessibility and the high reaction yield. By using the same conditions, *N*-glycolyl Lipid II **10** was also prepared in a satisfactory yield (85%; Table 1, entry **7**). With these encouraging results and valuable reaction parameters in hand, we sought to prepare *N*-glycolyl Lipid I. Notably, without adding UDP-GlcNAc, *N*-glycolyl Park's nucleotides **6** and **5** were converted to the corresponding NBD-*N*-glycolyl-Lipid I (**7**) and *N*-glycolyl-Lipid I (**8**). The reaction mixtures were extracted and purified by HPLC and characterized by ^{1}H NMR spectroscopy to give pure Lipid I **7** and **8** in good yields (75% and 80%, respectively; Table 1, entries 8 and 9). Our results indicate that the fluorescent probe attached to the ε-position of lysine of Park's nucleotide did not preclude the biosynthesis of Lipid I and Lipid II analogues.

Having studied the biocatalytic conditions in detail, new Park's nucleotides bearing a *N*-glycinyl group or different lengths of the peptide chain were designed and synthesized, and their conversion into the new corresponding Lipid I and Lipid II-based molecules was pursued. Following the similar protocol shown in Scheme 1, *N*-succinimidyl-2-trifluoroacetamidoacetate was utilized instead of *N*-succinimidyl acetoxyacetate to react with **1**. Two new fluorescent *N*-glycinyl Park's nucleotides **13** and **14** bearing the tripeptide and peptapeptide, respectively, were prepared (see the Supporting Information, Scheme S1). In addition, two new *N*-glycolyl Park's nucleotides (**11** and **12**) with a different length of the peptide chain were also prepared (Figure 3).

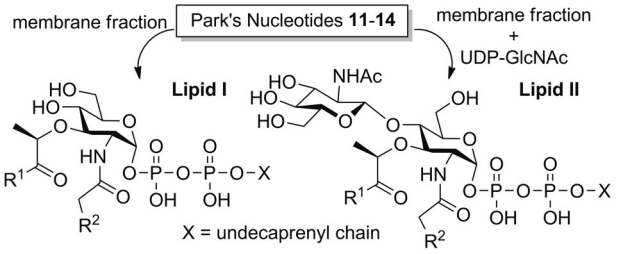


11: R^1 = L-Ala-D-Glu-L-Lys(NBD); R^2 = OH
12: R^1 = L-Ala; R^2 = OH
13: R^1 = L-Ala-D-Glu-L-Lys(NBD)-D-Ala-D-Ala; R^2 = NH_2
14: R^1 = L-Ala-D-Glu-L-Lys(NBD); R^2 = NH_2

Figure 3. Chemical structures of the peptide truncated *N*-glycolyl Park's nucleotides and *N*-glycinyl Park's nucleotides.

N-Glycolyl and *N*-glycinyl Park's nucleotides (**11**, **12**, **13**, and **14**) were submitted to the biocatalytic reactions previously developed (Table 1) and the results are shown in Table 2. *N*-Glycolyl Park's nucleotide **11** was converted to the corresponding Lipid I **15** and Lipid II **18** in yields of 68 and 67%, respectively. The use of *N*-glycolyl Park's nucleotide **12**, however, did not result in the detection of any desired products, suggesting that the major truncation of the peptide moiety in the substrate disabled the catalytic activity. Surprisingly, *N*-glycinyl Park's nucleotides **13** and **14** were converted to *N*-glycinyl Lipid I and Lipid II in high yields (Table 2, entries 3 and 4), proving that the amino group in **13** or **14** could be tolerated during the biocatalytic synthesis.

Table 2. Preparation of modified Lipid I and Lipid II through the biocatalytic method.



Entry	Park's nucleotide	R^1	R^2	Lipid I/ Yield [%]	Lipid II/ Yield [%]
1	**11**	L-Ala-D-Glu-L-Lys(NBD)	OH	**15**/68	**18**/67
2	**12**	L-Ala	OH	–/trace[a]	–/trace[a]
3	**13**	L-Ala-D-Glu-L-Lys(NBD)-D-Ala-D-Ala	NH_2	**16**/64	**19**/77
4	**14**	L-Ala-D-Glu-L-Lys(NBD)	NH_2	**17**/64	**20**/64

[a] Detected by mass spectrometry, not isolated.

With these structurally modified peptidoglycan precursors in hand, their biological activity toward PonA was investigated. *N*-Glycinyl Lipid II analogues **19** (pentapeptide) and **20** (tripeptide) were both found to be PonA substrates with similar substrate potency. Both were mostly consumed (> 80%) in 8 h during the transglycosylation reaction catalyzed by *Mtb* PonA, suggesting the presence of the last two amino acids (D-Ala-D-Ala) did not significantly affect the substrate-enzyme recognition[17] (see the Supporting Information, Figure S4). Kinetic studies revealed that the *N*-glycolyl Lipid II **9** and *N*-glycinyl Lipid II **19** have a similar binding affinity towards PonA with K_M values of 9.7 and 5.4 μM, respectively. Surprisingly, the k_{cat}/K_M values of **9** and **19** were higher than that of the bacterial *N*-acetyl Lipid II[16] ($1.06\times10^4\,M^{-1}s^{-1}$), indicating that the *N*-glycolyl or *N*-glycinyl group in Lipid II contributes more interactions with PonA than the *N*-acetyl group (Table 3; see the Supporting Information, Figure S5). Based on the peptidoglycan biosynthesis

Table 3. Kinetic parameters of *N*-acetyl Lipid II, **9** and **19** towards PonA.[a]

	N-acetyl-Lipid II[16]	**9**	**19**
K_M [μM]	26.4 ± 6.8	9.7 ± 1.5	5.4 ± 1.6
k_{cat} [s^{-1}]	0.28 ± 0.03	0.25 ± 0.01	0.23 ± 0.02
k_{cat}/K_M [$M^{-1}s^{-1}$]	1.06×10^4	2.57×10^4	4.26×10^4

[a] Experiments were performed in 0.085% decyl-PEG, Tris-HCl (50 mM), pH 8.0, $CaCl_2$ (10 mM), 10% DMSO, 15% MeOH and PonA (0.07 μM) at 25 °C and repeated in triplicate.

pathway, naturally occurring *N*-glycolyl Lipid I is located only on the cytoplasmic surface of the inner membrane, and therefore cannot interact with PonA. Unexpectedly, *N*-glycolyl Lipid I was found to be a PonA inhibitor with IC_{50} value of 295 μM (see the Supporting Information, Figure S6). For comparison purposes, undecaprenyl phosphate (C55P) and *N*-glycolyl Park's nucleotide (**6**) showed no inhibitory activity against PonA. These results suggest that a molecule containing the *N*-glycolyl-muramyl-pentapeptide pyrophosphate moiety linked with a hydrophobic lipid might play an important role for inhibitory activities against TGases.

In conclusion, a rapid and convenient method for the preparation of key precursors of mycobacterial cell walls, including *N*-glycolyl Lipid I and Lipid II, has been successfully developed. This new synthesis starts from diverse *N*-glycolyl Park's nucleotides, and proceeds through a biocatalytic synthesis. The biocatalyst sources, extracted from the crude membrane fraction of *M. flavus*, were used directly without further purification. Reaction optimization resulted in the development of protocols that are easy to perform and give satisfactory yields. Structurally new Park's nucleotides bearing peptide moieties of different lengths are well tolerated in this biocatalytic method. The unnatural *N*-glycinyl Lipid II, which bears a versatile amino functional group of potential utility in other chemical applications, was also found to be a substrate of PonA. Interestingly, *N*-glycolyl Lipid I was found to weakly inhibit TGase and is therefore a possible starting point for the development of new *Mtb* TGase inhibitors. The feasibility of this research approach is currently being investigated.

Experimental Section

General procedure for Lipid I and Lipid II preparation: UDP-GlcNAc (10 μmol, only for Lipid II preparation), *N*-glycolyl Park's nucleotide derivatives (1 μmol) and C55P (1.5 μmol) were dissolved in reaction buffer (containing Tris-HCl (10 mM), pH 8, $MgCl_2$ (0.5 mM) and 1% (w/v) Triton X-100). The cell-free membrane fraction (128 mg) from *Micrococcus flavus* was added into the reaction buffer and shaken at RT for 3 h. When Park's nucleotide was completely consumed, the crude reaction was extracted by 1-butanol and pyridine acetate. After centrifugation, the organic layer was collected and purified by RP-HPLC.

Acknowledgements

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Keywords: antibiotics · biocatalysis · inhibitors · synthetic methods · transglycosylase

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