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SYNTHETIC STUDIES ON *MYCOBACTERIUM TUBERCULOSIS* SPECIFIC FLUORESCENT PARK'S NUCLEOTIDE PROBE

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Dedicated to Professor Ryoji Noyori on the occasion of his 70th birthday

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Abstract - An efficient synthesis of *N*-glycolyl Park's nucleotide, a *Mycobacterium tuberculosis* (Mtb) specific peptidoglycan precursor, analog-fluorescein conjugate, is achieved. A fluorescent conjugated Mtb Park's nucleotide analog **5** would be a very useful probe for the characterization of *Mtb* MraY, catalyzing the biosynthesis of lipid I from UDP-*N*-acyl-Mur-pentapeptide, and for the development of high-throughput screening (HTS) for Mtb MraY inhibitors.

INTRODUCTION

Mycobacteria are classified as Gram-positive organisms, however, they have features of both Gram-positive and Gram-negative bacteria. The compositional complexity of the mycobacterial cell envelope differentiates *Mycobacterium* spp. from most other prokaryotes.[1] The cell envelope of *Mycobacterium tuberculosis* (Mtb) is made up of three major components: a plasma membrane, a covalently linked mycolic acid, arabinogalactan and peptidoglycan complex (MAPc), and a polysaccharide-rich capsule-like material. The unique nature of the MAPc led to the conclusion that the enzymes that biosynthesize this structure should yield a number of potentially unique drug targets. Peptidoglycan (PG) biosynthesis in *Mycobacterium* spp. has not been investigated in detail, but is assumed to be similar to that seen in *E. coli*.[2] This concept is supported by genetic analysis. Most of the genes involved in peptidoglycan biosynthesis in *E. coli* are known and orthologs have been identified in the Mtb genomes, except for the

genes responsible for the unique features of mycobacterial peptidoglycan. The biosynthesis of PG of *E. coli* has been discussed extensively in reviews by van Heijenoort.[2] Detailed analyses of the component of mycobacterial PG revealed that it contains a variety of modified molecules including an *N*-glycolyl (NGlyc) in addition to *N*-acetyl (NAc) group on the muramic acid (Mur), amidation of the carboxylic acids, and additional glycine or serine residues.[3] The *N*-glycolylation of sialic acids in eukaryotes is know to confer differential biological activities.[4] However, it is not know what role *N*-glycolylated muramic acid (MurGlyc) plays in peptidoglycan biology.[5] Recently, *namH* gene, encoding the hydroxylase responsible for the synthesis of UDP-MurNGlyc from UDP-MurNAc, was identified.[6] Because the *N*-glycolylated muramic acid is predominated in mycobacteria, it is of significance to characterize which PG biosynthetic enzymes (MurC, D, E, and F, and MraY) have better specificity against UDP-*N*-glycolylmuramic acid derived precursors of PG biosynthesis over their *N*-acetyl derivatives. In addition, *Mtb* MraY, catalyzing the transformation of UDP-*N*-acylmuramyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (Park's nucleotide)[7] to decaprenylpyrophosphoryl-*N*-acylmuramyl-L-Ala-γ-D-glu-*meso*-DAP-D-Ala-D-Ala (lipid I), is a target of interest for the discovery of novel antimycobacterial agents.[8] Thus, we are especially interested in characterizing the selectivity of *Mtb* MraY[9] against *N*-glycolyl-Park's nucleotide **1** (Figure 1). In addition, we have been developing a fluorescence polarization-based assay for *Mtb* MraY inhibitors which is 1) not dependent on radioactive substrates, and 2) amenable to high-throughput screening (HTS).

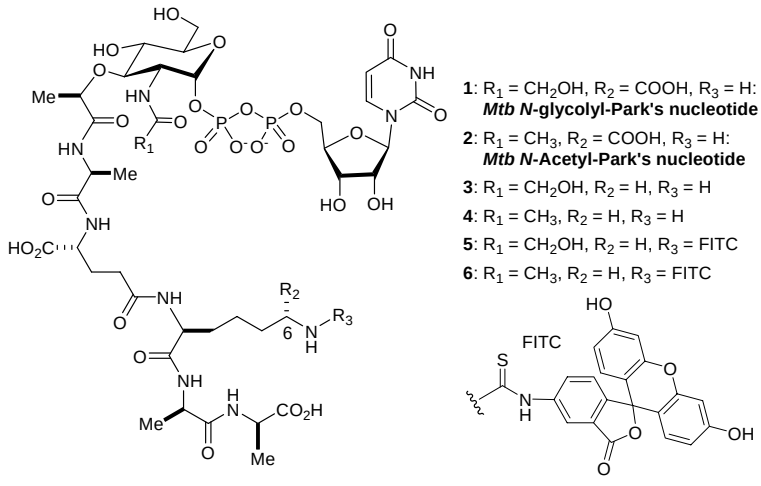


Figure 1. Structures of Mtb Park's nucleotides and their analogs.

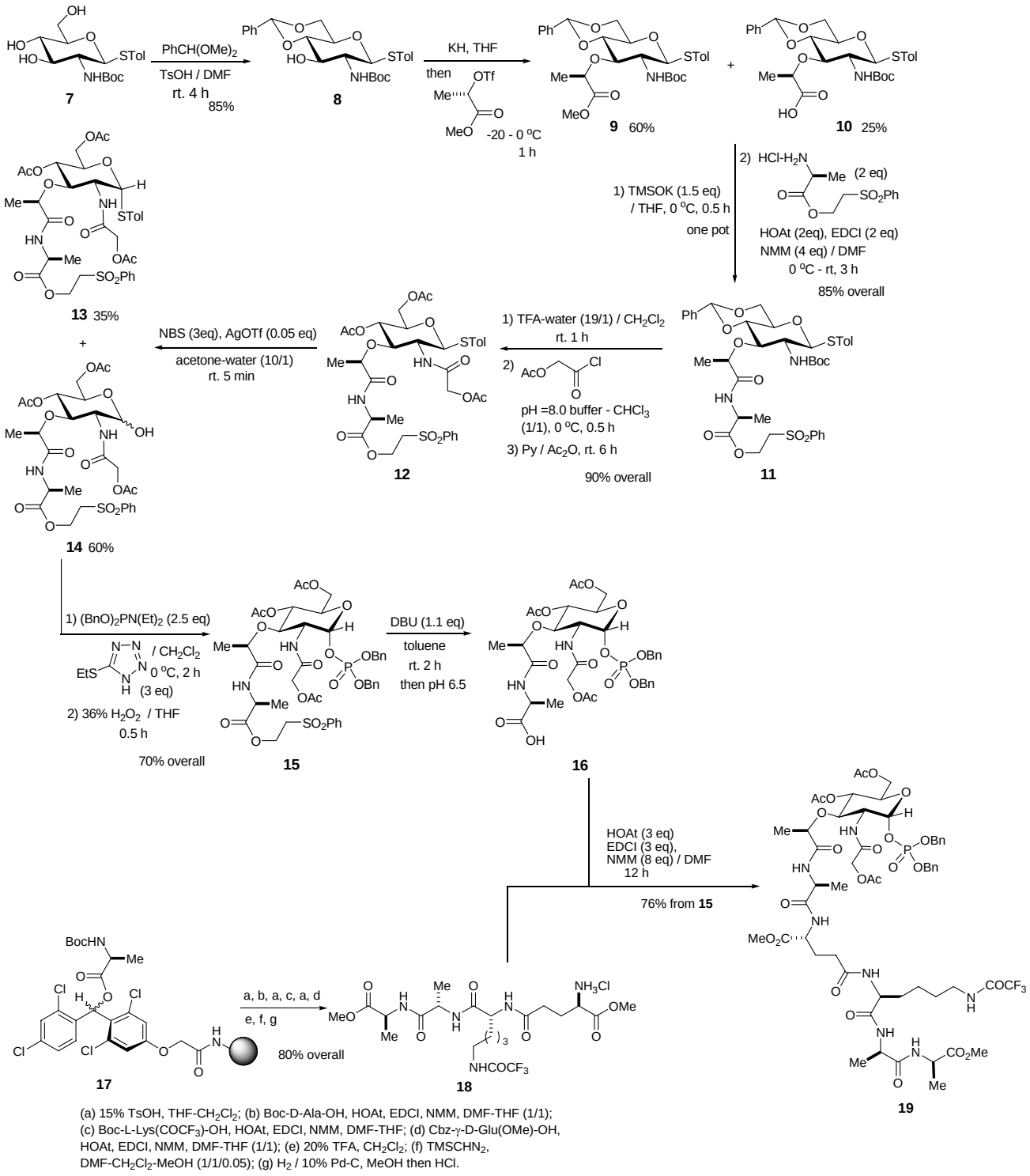
A requirement for *Mtb* MraY characterization and development of HTS is a significant supply of enzymatic substrate(s). It was reported that the dansylated (at R_3 position) derivative of **2** (Figure 1) was utilized in an assay using *E. coli* MraY and the dansylated lipid I was separated from the reaction mixtures; the *Km* for UDP-MurNAc-N^{ε}-dansylpentapeptide and UDP-MurNAc-pentapeptide **2** were 15

and 19 μM, respectively.[10] In addition, the lysine derivative **4** was demonstrated for the formation of the polymerized peptidoglycan using the PG biosynthetic enzymes *in vitro*.[11] Thus, the chemical modifications are tolerated at C6-*meso*-diaminopimelic acid (DAP) position of Park's nucleotide. We previously synthesized Mtb Park's nucleotide **2** efficiently through a chemoenzymatical process.[12] A total synthesis of **4**[13] was reported by Hitchcock and co-workers.[11] However, to date, no enzymatic or chemical synthesis of *N*-glycolyl-Park's nucleotide **1** or its lysine analog **3** has been reported. In the present work, we report a scalable route for the syntheses of **3** and its fluorescein-conjugate **5**.

RESULTS AND DISCUSSION

There are several drawbacks, from a practical point of view, in the reported synthesis of **4**; 1) poor selectivity (α/β = 2.5/1) of the α-phosphite formation reaction using $(BnO)_2PNEt_2$ and tetrazole,[11] 2) significantly slow rate (for 4~14 days in 30~50% yield)[14] of diphosphate forming reactions of α-glycosyl monophosphates with a commonly utilized 5'-UMP donor, uridine 5'-morpholinophosphonate[15], and 3) a time-consuming purification of a tetrasodium diphosphoryl-dicarboxylate which cannot be applied to a large-scale purification.

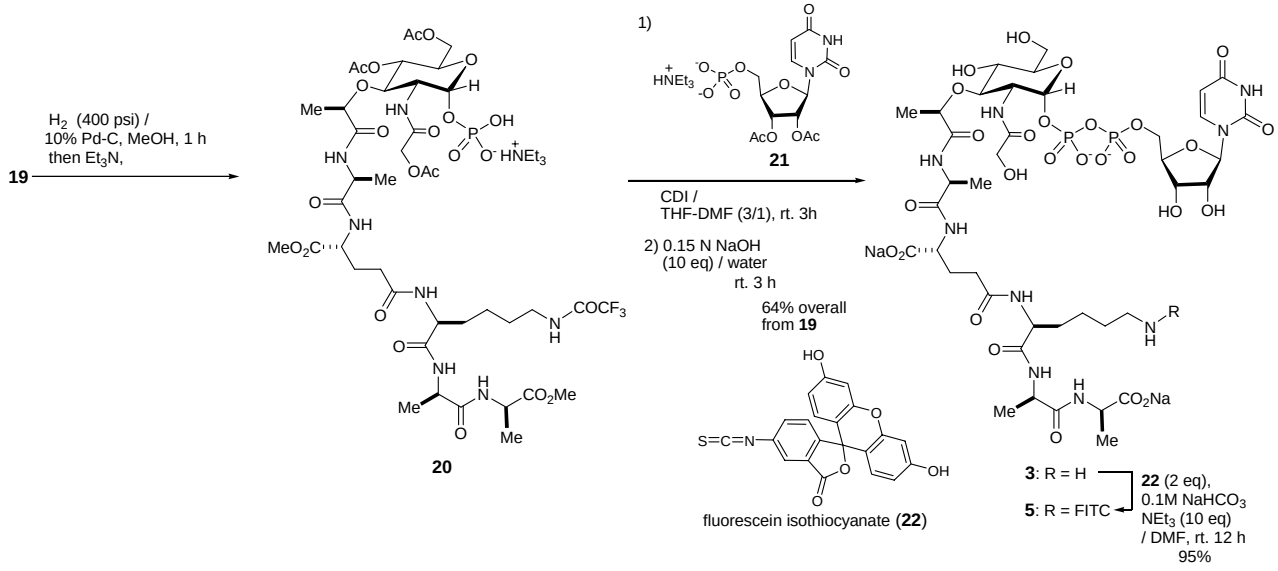
Commercially available carbohydrate building block for the synthesis of *N*-acetylmuramic acid (MurNAc), benzy 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[16] is relatively expensive for a hundred-gram quantity and its conversion to a *N*-glycolyl derivative requires additional several steps including a protecting group shuffle. Thus, the *p*-tolylthioglycoside **7**, which is readily synthesized from GlcNAc peracetate in three steps,[17] was chosen as a starting material for the synthesis of *N*-glycolyl-Park's nucleotide derivatives (Scheme 1). The regioselective benzylidene acetalization of 4,6-diols of **7** using benzaldehyde dimethylacetal and a catalytic amount of anhydrous TsOH provided **8** without loss of the Boc group. The C3-hydroxyl group of **8** was reacted with an activated methyl lactate, (*S*)-methyl 2-(trifluoromethylsulfonyloxy)propanoate, in the presence of KH to furnish a mixture of the ester ether **9** and its free carboxylic acid **10** in 60% and 25%, respectively. A mixture of **9** and **10** was subjected to TMSOK under an anhydrous condition to provide the potassium carboxylate. The coupling reaction with HCl•H-L-Ala-$O(CH_2)_2SO_2Ph$ was performed in a one-pot operation using HOAt (1-hydroxy-7-azabenzotriazole), EDCI, and NMM in a mixture of THF and DMF (1/1) solution to give rise to **11** in 85% overall yield.[18] The benzylidene acetal and Boc groups in **11** were simultaneously deprotected with aq. TFA and the deprotected C2-amine was selectively acylated with acetoxyacetyl chloride *via* a Schotten-Baumann reaction, and diols at the C4 and C6 positions were acetylated (90% overall yield for **12**).



Scheme 1. Synthesis of the intermediate **19**.

Deprotection condition of the anomeric protecting group of **12** has not been optimized but could conveniently be regenerated to the anomeric free alcohol **14** in 60% yield by using NBS in the presence of a catalytic amount of AgOTf within 5 minutes.[19] α-Selective phosphite formation of **14** followed by oxidation to form phosphate were carried out through a Wong's protocol using dibenzyl *N,N*-

diethylphosphoramidite.[20] However, the phosphite forming reaction with an established base,[21] 1-*H*-tetrazole, did not provide the desired product reproducibly and the α/β-selectivity was very poor (~3/1). On the other hand, the same reaction with 5-(ethylthio)-1*H*-tetrazole[22] gave the α-phosphate **15**, after oxidation with aq. H_2O_2, solely as the desired isomer in 70% overall yield.[23] Deprotection of the 2-(phenylsulfonyl)ethanol protecting group of **15** was achieved by the treatment with DBU to furnish the α-phosphoryl-MurGlyc-monopeptide building block **16**.

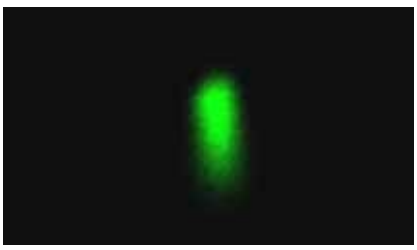


Scheme 2. Syntheses of **3** and **5**.

The appropriately protected tetrapeptide, D-isoglutamyl-L-lysinyl-D-alanyl-D-alanine **18**, was synthesized using the Boc-strategy on an acid and base stable hydroxytetrachlorodiphenylmethyl (HTPM) linker resin.[24] Three grams of the Boc-D-Ala-HTPM resin (~1.0 mmol/g) were used for the following sequential ligation reactions with Boc-D-Ala-OH, Boc-L-Lys($COCF_3$)-OH, and Cbz-γ-D-Glu(OMe)-OH. The generated tetrapeptide on HTPM resin was cleaved with 20% TFA in CH_2Cl_2 followed by methylation with $TMSCHN_2$ under an optimized condition (DMF-CH_2Cl_2-MeOH) afforded Cbz-γ-D-Glu(OMe)-L-Lys($COCF_3$)-D-Ala-D-Ala-OMe in 80% overall yield after SiO_2-plug filtration ($CHCl_3$:MeOH=3:1). Hydrogenolysis of the Cbz group of the tetrapeptide followed by the treatment with HCl saturated in MeOH provided HCl•H-γ-D-Glu(OMe)-L-Lys($COCF_3$)-D-Ala-D-Ala-OMe (**18**). Coupling of the α-phosphoryl MurGlyc-monopeptide **16** with excess of **18** (2~3 eq) under a condition of HOAt, EDCI, and NMM yielded the α-phosphoryl MurGlyc-pentapeptide **19** in over 80% yield. Hydrogenolitic debenzylations of **19** followed by the treatment with Et_3N (excess) resulted in the corresponding monotriethylammonium phosphate **20** in quantitative yield, whose structure was established by ^{1}H-NMR

analysis (Scheme 2). As described above UDP-glycoside synthesis using uridine 5'-morpholinophosphonate[11,14] is typically a slow process and often resulted in unsatisfactory yield. Thus, triethylammonium 5'-UMP diacetate **21**[25] was introduced for a carbonyldiimidazole (CDI) promoted diphosphate-formation reaction. 5'-UMP diacetate **21** was first activated with CDI and the excess CDI was quenched with MeOH to afford 1*H*-imidazole-1-carboxylic (phosphoric) anhydride and methyl 1*H*-imidazole-1-carboxylate[26]. All volatiles were extensively removed and the remaining mixture was subjected to cross-coupling reaction with the monotriethylammonium α-phosphoryl MurNGlyc-pentapetide **20**. The reaction was completed within 3 h and the excess reagents and CDI derived by-products were removed by passing a sephadex column. Global deprotection of fully-protected UDP-MurGlyc-pentapeptide with aq. NaOH and purification by a sephadex LH-20 chromatography [0.05 M NH_4HCO_3-CH_3CN (3/1)] afforded **3** as a white powder in 64% overall yield from **19**. The structure of **3** was confirmed by ^{1}H- and ^{13}C-NMRs, negative ESI-TOF-MS spectroscopy, and chromatographic behavior. Fluorescein could efficiently be conjugated to the lysine residue of **3**; the reaction of **3** with fluorescein isothiocyanate (FITC, **22**) in the presence of Et_3N in 0.1 M $NaHCO_3$-DMF (1/2) furnished **5** as an orange powder in 95% yield.[27]

As a preliminary experiment we conducted the fluorescein uptake studies using *Mycobacterium bovis* BCG. *M. bovis* BCG was treated with **5** and time course of fluorescein uptake by *M. bovis* BCG was monitored after an extensive wash of bacteria *via* a confocal laser scanning microscope. Intensity of fluorescence was variable within each time point. The intensely fluorescent bacteria were observed within 3h, and fluorescence remains for at least 6h (Figure 2). These observations indicate that the fluorecein-**3** conjugate can be transferred into the cytosol of the bacteria and **5** in the cytosol may be utilized as a building block for the biosynthesis of PG biosynthetic precursors.



The image obtained *via* a Zeiss LSM Meta
510 confocal microscope with 100x objective lens.

Figure 2. Fluorescent imaging of *M. bovis* BCG, after the treatment of **5** for 3h.

CONCLUSION

In conclusion, chemical synthesis of Mtb *N*-glycolyl-Park's nucleotide analogs summarized in Scheme 1 and 2 includes 1) an efficient synthesis of α-phosphoryl-MurGlyc-monopeptide **16**, 2) high-yielding synthesis of oligopeptide on the HTPM resin, 3) expeditious synthesis of UDP-glycoside using a

modified 5'-UMP derivative **21**, and 4) a reliable purification method for Park's nucleotide derivatives which is amenable to a larger-scale purification. Although the synthetic scheme demonstrated here is continuing to increase the yields for steps **12** → **14** → **15**, the synthetic process for the preparation of **3** and **5** in Scheme 1 and 2 is operationally simple and does not require sophisticated chromatographic purifications. Chemical synthesis of Parks' nucleotide derivatives demonstrated here serves as a complemental manner; chemoenzymatic synthesis provides Mtb *N*-acetyl-Park's nucleotide **2** and its fluorescent probes, whereas chemical synthesis furnishes *N*-glycolyl-Park's nucleotide derivatives.

Characterization of *Mtb* MraY using the substrates **2**, **3**, **4**[28], **5** and **6**, and development of HTS against *Mtb* MraY using **5** will be reported elsewhere.

EXPERIMENTAL

General Considerations. All glassware was oven dried, assembled hot and cooled under a stream of nitrogen before use. Reactions with air sensitive materials were carried out by standard syringe techniques. Commercially available reagents were used as received without further purification. Thin layer chromatography was performed using 0.25 mm silica gel 60 (F254, Merck) plates visualizing at 254 nm, or developed with potassium permanganate solutions by heating with a hot-air gun. Specified products were purified by flash column chromatography using silica gel 60 (230-400 mesh, Merck). IR absorptions on NaCl plates were run on a Perkin Elmer FT-IR 1600. ^{1}H NMR spectral data were obtained using Varian 300, 400 or 500 MHz instruments. The residual solvent signal was utilized as an internal reference. ^{13}C NMR spectral data were obtained using a Varian 75 or 100 or 125 MHz spectrometer. Chemical shifts were reported in parts per million (ppm) downfield from TMS, using the middle resonance of $CDCl_3$ (77.0 ppm) as an internal standard. For all NMR spectra, δ values are given in ppm and *J* values in Hz. Mass spectra were obtained at Colorado State University's Central Instrument Facility. Optical rotations were taken using Rudolph research–Autopol III, automatic polarimeter.

Synthesis of tolyl 2-(tert-butoxycarbonylamino)-2-deoxy-4,6-O-(phenylmethylene)-1-thio-β-D-glucopyranoside (8). To a stirred solution of **7** (3.0 g, 7.78 mmol) in DMF (30 mL) was added $PhCH(OMe)_2$ (3.55g, 23.3 mmol) and TsOH (133 mg, 0.77 mmol). The reaction mixture was stirred at rt for 6 h and quenched with aq. $NaHCO_3$. The water phase was extracted with EtOAc and the combined organic phase was washed with brine, dried over Na_2SO_4, and evap. in *vaccuo*. Purification by silica gel chromatography (hexanes/$CHCl_3$/EtOAc=10/10/2) furnished **8** (3.13 g, 85%). Data for **8**: mp 215-216 ^{o}C; $[\alpha]^{20}_D$ -31.0 (*c* 0.57, $CHCl_3$); IR (film) 3320 cm^{-1}, 1686, 1532; ^{1}H NMR (400 MHz, $CDCl_3$); 7.48 (m, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.35 (m, 3H), 7.14 (d, *J* = 8.0 Hz, 2H), 5.54 (s, 1H), 4.87 (broad, 2H), 4.36 (m, 1H), 4.04 (broad, 1H), 3.78 (t, *J* = 9.6 Hz, 1H), 3.51 (m, 3H), 3.30 (broad, 1H), 2.36 (s, 3H), 1.49 (s,

9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.30, 138.88, 137.20, 133.74, 130.04, 129.44, 128.51, 126.53, 102.01, 86.97, 81.43, 80.94, 73.16, 70.52, 68.76, 57.23, 28.53, 2140; HRMS (ESI) Calcd. for $C_{25}H_{31}NO_6NaS$ $(M+Na)^+$: 496.1764; found: 496.1769.

Synthesis of tolyl 2-(tert-butoxycarbonylamino)-2-deoxy-3-O-(2-methoxy-1S-methyl-2-oxoethyl)-4,6-O-(phenylmethylene)-1-thio-β-D-glucopyranoside (9). To a solution of alcohol **8** (2.00 g, 4.25 mmol) in dry THF (20 mL) at -20°C was added KH (washed with dry hexanes and dried under high vacuum, 0.19 g, 4.68 mmol) and the reaction mixture was stirred for 30 min. at -20 °C. 2-(*S*)-Trifluoromethanesulfonyloxy-propionic acid methyl ester (2.01 g, 8.5 mmol) in dry THF (5 mL) was added to the above solution over 5 min. The reaction mixture was slowly warmed to 0° C over 1 h and quenched by sat. aq. NH_4Cl. The water phase was extracted with $CHCl_3$ and combined extracts were dried over Na_2SO_4. Purification by silica gel chromatography (Hexanes/$CHCl_3$/EtOAc=10/10/2 to $CHCl_3$/MeOH=5/1) gave **9** (1.42 g, 60%) and gave acid **10** (0.58 g, 25%). Data for **9**: mp 205-206 °C; $[\alpha]^{20}_D$ +4.8 (*c* 0.50, $CHCl_3$); IR (film) 1687 cm^{-1}, 1535, 1095; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44-7.36 (m, 6H), 7.11 (d, *J* = 8.0 Hz, 2H), 5.54 (s, 1H), 5.33 (d, *J* = 6.8 Hz, 1H), 5.02 (m, 1H), 4.43 (m, 1H), 4.33 (dd, J_1 = 4.8 Hz, J_2 = 10.4 Hz, 1H), 4.00 (m, 1H), 3.78 (t, *J* = 10.0 Hz, 1H), 3.71 (s, 3H), 3.60 (m, 1H), 3.46 (m, 1H), 3.29 (m, 1H), 2.34 (s, 3H), 1.48 (s, 9H), 1.38 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.00, 155.98, 138.28, 137.38, 133.30, 129.90, 129.19, 128.48, 126.07, 101.30, 88.18, 82.31, 79.85, 78.70, 76.00, 70.37, 68.82, 56.37, 52.16, 28.59, 21.34, 19.17; HRMS (ESI) Calcd. for $C_{29}H_{37}NO_8NaS$ $(M+Na)^+$: 582.2143; found: 582.2130.

Synthesis of (2S)-2-(tolyl 2-(tert-butoxycarbonylamino)-2-deoxy-4,6-O-(phenylmethylene)-β-D-glucopyranosid-3-O-yl)-N-((phenylsulfonyl)ethoxy-1S-methyl-2-oxoethyl)-propionamide (11). To a stirred solution of **9** and **10** (1.42 g, ~2.55 mmol) in dry THF (7.5 mL) at 0 °C was added TMSOK (90%, 0.54 g, 3.8 mmol). The reaction mixture was stirred for 30 min. at 0 °C and dry DMF (10 mL) was added. Into the reaction mixture HCl•H-L-Ala-O$(CH_2)_2SO_2Ph$ (2.12 g, 7.22 mmol), HOAt (1-hydroxy-7-azabenzotriazole) (0.99 g, 7.22 mmol), EDCI HCl (1.39 g, 7.22 mmol) and *N*-methylmorpholine (1.46 g, 14.4 mmol) were added at 0 °C. The reaction mixture was warm to rt over 3 h and quenched with H_2O. The water phase was extracted with $CHCl_3$, and the combined extracts were dried over Na_2SO_4, and evap. in *vacuo*. Purification by silica gel chromatography ($CHCl_3$/MeOH=10/1) gave **11** (2.41 g, 85%). Date for **11**: mp 217-218 °C; $[\alpha]^{20}_D$ + 4.5 (*c* 0.40, $CHCl_3$); IR (film) 1688 cm^{-1}, 1532, 1145, 771; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.0 Hz, 2H), 7.68 (m, 1H), 7.56 (m, 2H), 7.42 (m, 2H), 7.35 (m, 5H), 7.08 (d, *J* = 8.0 Hz, 2H), 5.52 (s, 1H), 5.20 (d, *J* = 8.4 Hz, 1H), 4.99 (d, *J* = 9.6 Hz, 1H), 4.47 (m, 3H), 4.33 (dd, J_1 = 5.2 Hz, J_2 = 10.4 Hz, 1H), 4.22 (m, 2H), 3.95 (t, *J* = 9.2 Hz, 1H), 3.77 (t, *J* = 10.4 Hz, 1H), 3.58 (t, *J*

= 8.8 Hz, 1H), 3.47 (m, 4H), 2.32 (s, 3H), 1.45 (s, 9H), 1.37 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.30, 172.08, 155.65, 139.06, 138.48, 137.17, 134.38, 133.16, 129.97, 129.72, 129.30, 129.08, 128.52, 128.28, 126.09, 101.45, 87.99, 81.78, 80.71, 79.98, 78.43, 70.22, 68.74, 58.12, 56.65, 55.14, 48.09, 28.60, 21.35, 19.55, 17.55; HRMS (ESI) Calcd. for $C_{39}H_{48}N_2O_{11}NaS_2$ $(M+Na)^+$: 807.2603; found: 807.2603.

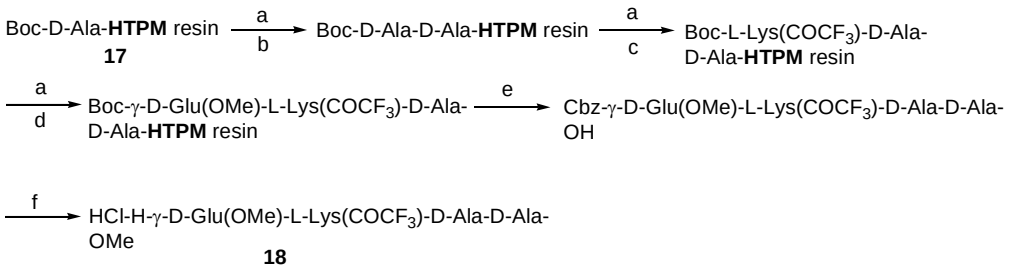
Synthesis of (2S)-2-(tolyl 2-(2-acetoxyacetamido)-4,6-di-O-acetyl-2-deoxy-β-D-glucopyranosid-3-O-yl)-N-((phenylsulfonyl)ethoxy-1S-methyl-2-oxoethyl)-propionamide (12). To a solution of **11** (0.39 g, 0.50 mmol) in CH_2Cl_2 (4 mL) was added TFA/H_2O (19/1, 2 mL) at rt. After 1 h, TFA was removed under high vacuum. The crude product was dissolved in an aqueous buffer (K_2CO_3/HOAc, pH=8.0, 2 mL) and $CHCl_3$ (2 mL), and acetoxyl acetic chloride (0.27 g, 2 mmol) was added in the reaction mixture at 0 °C. After 30 min. at 0 °C, the reaction mixture was extracted with $CHCl_3$ and the combined extracts were dried over Na_2SO_4, and evap. *in vaccuo* to give the crude product. This was dissolved in pyridine (2 mL) and acetic anhydride (1 mL). After 6 h at rt, all volatiles were removed under high vacuum at 30 °C. Purification by silica gel chromatography gave **12** (0.35 g, 90%). Date for **12**: $[\alpha]^{20}_D$ -6.7 (*c* 0.58, $CHCl_3$); IR (film) 2360 cm^{-1}, 1746, 1225; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.95 (d, *J* = 7.3 Hz, 2H), 7.71 (m, 1H), 7.62 (m, 2H), 7.38 (d, *J* = 8.1 Hz, 2H), 7.10 (d, *J* = 8.1 Hz, 2H), 7.01 (d, *J* = 7.2 Hz, 1H), 6.69 (d, *J* = 8.1 Hz, 1H), 5.11 (d, *J* = 10.5 Hz, 1H), 4.95 (t, *J* = 9.6 Hz, 1H), 4.61-4.43 (m, 4H), 4.24-4.12 (m, 4H), 4.03 (dd, J_1 = 6.6 Hz, J_2 = 14.1 Hz, 1H), 3.66 (m, 1H), 3.46 (m, 3H), 2.35 (s, 3H), 2.20 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H) 1.35-1.27 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.56, 172.01, 170.85, 170.10, 169.79, 168.27, 139.09, 138.60, 134.40, 133.48, 129.84, 129.71, 128.20, 85.35, 79.51, 78.14, 75.92, 69.89, 63.12, 62.70, 58.22, 56.15, 54.99, 48.21, 21.29, 21.03, 20.93, 20.80, 18.91, 16.97; HRMS (ESI) calcd. for $C_{35}H_{45}N_2O_{14}S_2$ $(M+H)^+$: 781.2307; found: 781.2307.

Synthesis of (2S)-2-(tolyl 2-(2-acetoxyacetamido)-4,6-di-O-acetyl-2-deoxy-1-(O,O-dibenzylphosporyl)-α-D-glucopyranosid-3-O-yl)-N-((phenylsulfonyl)ethoxy-1S-methyl-2-oxoethyl)-propionamide (15). To a stirred solution of **12** (150 mg, 0.19 mmol) in acetone/H_2O (10/1, 2 mL) was added AgOTf (2.4 mg, 0.01 mmol), NBS (0.10 g, 0.57 mmol). After 5 min. at rt, the reaction was quenched with sat. aq. Na_2SO_3. The water phase was extracted with $CHCl_3$ and combined extracts were dried over Na_2SO_4. Purification by silica gel chromatography ($CHCl_3$/MeOH = 30/1 to 20/1) gave **14** (77 mg, yield 60%) and **13**. This was dissolved in dry CH_2Cl_2 (2 mL) and 5-ethylthio-1*H*-tetrazole (42.2 mg, 0.33 mmol), dibenzyl *N*,*N*-diethylphosphoramidite (88.8 mg, 0.28 mmol) were added at 0 °C. After 2 h at 0 °C, the reaction mixture was quenched with sat. aq. $NaHCO_3$. The water phase was extracted with $CHCl_3$ and combined extracts were dried over Na_2SO_4, and evap. in *vaccuo*. To a stirred solution of the crude phosphite in THF (4 mL)

was added $NaHCO_3$ (20 mg), then H_2O_2 (36%, 0.1 mL) at -15 °C. The reaction mixture was warmed to 0 °C for 30 min and quenched with sat. aq. Na_2SO_3 at the same temperature. The water phase was extracted with $CHCl_3$ and the combined extracts were dried over Na_2SO_4. Purification by silica gel chromatography ($CHCl_3$/MeOH=40/1) gave **15** (71.9 mg, 70%). Data for **15**: mp 52-53 °C; $[\alpha]^{20}_D$ +20.5 (*c* 0.40, $CHCl_3$); IR (film) 1746 cm^{-1}, 1225, 1144, 1042; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (m, 2H), 7.67 (m, 1H), 7.58 (t, *J* = 8.0 Hz, 2H), 7.33 (m, 10H), 7.20 (d, *J* = 8.0 Hz, 1H), 6.69 (d, *J* = 6.8 Hz, 1H), 5.69 (dd, J_1 = 3.2 Hz, J_2 = 6.0 Hz, 1H), 5.09-4.99 (m, 5H), 4.44 (t, *J* = 6.0 Hz, 2H), 4.35 (d, *J* = 2.0 Hz, 2H), 4.27 (m, 1H), 4.20 (m, 1H), 4.08 (dd, J_1 = 4.4 Hz, J_2 = 12.8 Hz, 1H), 3.93 (m, 3H), 3.60 (t, *J* = 9.6 Hz, 1H), 3.40 (m, 2H), 2.05 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H), 1.29 (d, *J* = 7.2 Hz, 3H), 1.26 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 172.17, 171.66, 170.84, 169.90, 169.35, 168.29, 139.24, 135.86, 134.40, 129.72, 129.19, 128.97, 128.32, 128.28, 96.46 (d, *J* = 5.5 Hz),78.14, 76.48, 70.42, 70.26, 70.20, 69.24, 62.86, 61.66, 58.29, 55.01, 53.15, 48.24, 21.03, 20.88, 20.75, 19.02, 17.35; HRMS (ESI) calcd. for $C_{42}H_{51}N_2O_{18}NaPS$ $(M+Na)^+$: 957.2498; found: 957.2483.

(2S)-2-(Tolyl 2-(2-acetoxyacetamido)-4,6-di-O-acetyl-2-deoxy-α-D-glucopyranosid-3-O-yl)-N-((phenylsulfonyl)ethoxy-1S-methyl-2-oxoethyl)-propionamide (13). Data for **13**: ^1H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 7.2 Hz, 2H), 7.75 (d, *J* = 8.0 Hz, 2H), 7.71 (m, 1H), 7.40 (m, 2H), 7.34 (d, 7.6 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 2H), 6.52 (d, *J* = 8.0 Hz, 1H), 5.72 (d, *J* = 3.6 Hz, 1H), 5.04 (t, *J* = 9.6 Hz, 1H), 4.61 (m, 2H), 4.50 (m, 2H), 4.30 (m, 1H), 4.14 (m, 2H), 3.94 (dd, J_1 = 2.0 Hz, J_2 = 12.4 Hz, 1H), 3.83 (m, 1H), 3.71 (t, *J* = 9.6 Hz, 1H), 3.59-3.50 (m, 3H), 2.43 (s, 3H), 2.17 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H), 1.42 (d, *J* = 7.2 Hz, 3H), 1.31 (d, *J* = 6.8 Hz, 3H), 1.26 (m, 2H). LRMS (ESI) $C_{35}H_{44}N_2O_{14}S_2$ found 803 $(M+Na^+)$.

Synthesis of HCl-H-γ-D-Glu(OMe)-L-Lys($COCF_3$)-D-Ala-D-Ala-OMe (18) using Boc-D-Ala-HTPM resin.



a) 20% TsOH/THF-CH_2Cl_2 (1/1), 6 h.; b) Boc-D-Ala-OH, EDCI, HOAt, NMM / DMF-THF (1/1), 6 h, c) Boc-L-Lys($COCF_3$)-OH, EDCI, HOAt, NMM / DMF-THF (1/1), 6 h.; d) Cbz-γ-D-Glu(OMe)-OH, EDCI, HOAt, NMM / DMF-THF (1/1), 6 h.; e) 20% TFA in CH_2Cl_2, 1 h.; f) $TMSCH_2N_2$ / DMF-MeOH-CH_2Cl_2

(1/0.05/1); g) H_2 (400 psi). 10%-Pd-C / MeOH then HCl saturated in MeOH; overall yield 80%. Data for **18**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.25 (broad, 1H), 8.42 (d, *J* = 7.2 Hz, 1H), 8.27 (d, *J* = 7.2 Hz, 2H), 7.23-7.10 (m, 3H), 4.41 (m, 2H), 4.22 (t, *J* = 4.4 Hz, 1H), 4.13 (t, *J* = 6.2 Hz, 1H), 3.84 (s, 3H), 3.69 (s, 3H), 2.50 (t, *J* = 7.8 Hz, 2H), 2.26 (m, 2H), 2.08 (m, 2H), 1.81-1.68 (m, 4H), 1.60 (m, 2H), 1.42 (d, *J* = 7.2 Hz, 3H), 1.37 (d, *J* = 7.2 Hz, 3H). HRMS (ESI) Calcd. for $C_{19}H_{31}ClF_3N_5NaO_8$ $(M+Na)^+$: 572.1711; found: 572.1718.

Synthesis of α-phospho-MurNGlyc-L-Ala-γ-D-Glu(OMe)-L-Lys(COCF$_3$)-D-Ala-D-Ala-OMe, 19.

To a stirred solution of **15** (30 mg, 0.03 mmol) in dry toluene (2 mL) was added DBU (5 mg, 0.03 mmol) at rt. After 2 h, the reaction mixture was quenched with aq. NH_4Cl (pH= 6.5). The water phase was extracted with $CHCl_3$ and the combined extracts were dried over Na_2SO_4, and evap. in *vacuo*. The crude product was dissolved in DMF (2 mL) and **18** (36.9 mg, 0.06 mmol), HOAt (13 mg, 0.09 mmol), EDCI (20 mg, 0.09 mmol), and *N*-methylmorpholine (28 mg, 0.24 mmol) were added at rt. After 3 h, the reaction mixture was quenched with aq. 10% $NaHCO_3$. The water phase was extracted with $CHCl_3$ and the combined extracts were dried over Na_2SO_4. Purification by silica gel chromatography ($CHCl_3$/MeOH/Et_3N = 200/10/1) gave the α-phosphoryl-MurNGlyc-pentapeptide **19** (29.4 mg, 76%). Data for **19**: $[\alpha]^{20}_D$ +22.5 (*c* 0.28, H_2O); IR (film) 1747, 1670cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53-7.40 (m, 3H), 7.37 (m, 10H), 7.18 (d, *J* = 5.6 Hz, 1H), 7.11 (d, *J* = 7.2 Hz, 1H), 7.01 (d, *J* = 6.4 Hz, 1H), 5.75 (dd, J_1 = 2.8 Hz, J_2 = 5.6 Hz, 1H), 5.07 (m, 4H), 2.55-4.42 (m, 5H), 4.32 (m, 3H), 4.14 (dd, J_1 = 4.0 Hz, J_2 = 12.4 Hz, 1H), 4.07 (m, 1H), 3.95 (m, 2H), 3.74 (s, 3H), 3.71 (s, 3H), 3.65 (t, *J* = 9.6 Hz, 1H), 3.65 (t, *J* = 9.6 Hz, 1H), 3.36 (m, 3H), 2.40 (m, 1H), 2.26 (m, 1H), 2.19 (m, 1H), 2.12 (s, 3H), 2.08 (s, 3H), 2.02 (s, 3H), 1.85 (m, 4H), 1.45 (m, 3H), 1.43 (m, 12H), 1.32 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.60, 173.12, 172.87, 172.41, 172.30, 170.86, 170.45, 169.48, 168.70, 157.75 (d, *J* = 26.6 Hz, $\underline{CO}CF_3$), 135.40, 135.24, 129.67, 129.28, 129.05, 128.41, 128.38, 128.16, 116.25 (d, *J* = 286.0 Hz, $CO\underline{CF_3}$), 96.48, 76.34, 70.37, 69.20, 62.81, 61.66, 54.10, 53.54, 52.77, 52.61, 51.03, 50.41, 49.39, 48.33, 46.00, 39.52, 31.63, 31.08, 28.39, 27.73, 22.47, 21.07, 20.90, 20.78, 18.79, 18.10, 17.68, 17.68; HRMS (ESI) Calcd. for $C_{55}H_{76}N_7O_{23}F_3P$ $(M+H)^+$: 1290.4677; found: 1290.4647.

Synthesis of triethylammonium (2R,3R,4R,5R)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-5-(phosphonooxymethyl)tetrahydrofuran-3,4-diyl diacetate (triethylammonium 5'-UMP diacetate, 21). To a stirred solution of uridine (4.0 g, 16.3 mmol) in pyridine (10 mL) was added Ac_2O (10 mL). After 12 h, all volatiles were evaporated. The crude product was azeotroped from toluene. This was dissolved in abs. MeOH and $[^tBu_2SnOH]_2$ (572 mg, 1.14 mmol) was added. The reaction mixture was stirred for 6 h, and

all volatiles were evaporated. The crude product was passed though SiO_2-plug (EtOAc) to provide (*2R,3R,4R,5R*)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diyl diacetate (4.27 g, 13.04 mmol). To a stirred solution of uridine diacetate (1.0 g, 3.04 mmol) in CH_2Cl_2 (15 mL) was added triazole (421 mg, 6.10 mmol) and $(BnO)_2PNEt_2$ (90%, 9.15 mmol) were added at 0 ^oC. After 15 min, the reaction was quenched with aq. $NaHCO_3$, and the water phase was extracted with CH_2Cl_2. The combined extracts were washed with brine, dried over Na_2SO_4, and evap. *in vaccuo*. The crude phosphite was oxidized with 36% H_2O_2 in THF (1/5) at 0 ^oC for 30 min. Excess H_2O_2 was carefully quenched with aq. $NaHCO_3$ and $NaSO_3$ at 0 ^oC. Purification by silica gel chromatography (EtOAc/hexanes =2/1 to EtOAc) provided uridine 5'-dibenzylphophate (1.51 g, 2.58 mmol). To a stirred solution of uridine 5'-dibenzylphophate (1.51 g, 2.58 mmol) in MeOH under N_2 was added 10% Pd-C (150 mg). H_2 was introduced by using a double-folded balloon. After 1 h, the reaction mixture was filtered and added Et_3N (5.16 mmol). All volatiles were evaporated in vaccuo to provide **21** (quantitative yield based on ^{1}H-NMR analysis). Data for **21**: ^{1}H-NMR (CD_3OD, 400 MHz): δ 1.31 (t, *J* = 7.2 Hz, 9H), 2.04 (s, 3H), 2.13 (s, 3H), 3.18 (q, *J* = 7.2 Hz, 6H), 4.12 (d, *J* = 7.2 Hz, 2H), 4.99 (bs, 3H), 5.42 (t, *J* = 6.0 Hz, 1H), 5.49 (bs, 1H), 5.82 (d, *J* = 8.0 Hz, 1H), 6.15 (d, *J* = 6.8 Hz, 1H), 8.04 (d, *J* = 8.0 Hz, 1H); ^{13}C-NMR (CD_3OD, 100MHz) δ 9.3, 20.4, 20.7, 47.6, 65.8, 73.1, 74.6, 83.8, 83.9, 87.5, 103.9, 142.5, 152.6, 166.0, 171.2, 171.5. HRMS calcd. for $C_{19}H_{32}N_3O_{11}P$: 509.1774: found: 509.1779. The steps 3 and 4 in the synthesis of **21** could be replaced by the treatment with $POCl_3$ (1.1 eq) in CH_2Cl_2-pyridine followed by hydrolysis with Et_3N/water. This procedure requires an ion-exchange chromatography to result in ~55% yield.

Synthesis of uridine 5'-diphospho-MurNGly-L-Ala-γ-D-Glu(ONa)-L-Lys-D-Ala-D-Ala-ONa, 3.

To the solution pentapeptide (5.0 mg, 3.88 μmol) in MeOH (1 mL) was added Pd/C (10%, 3 mg) under nitrogen atmosphere at rt. The switch of nitrogen atmosphere to hydrogen atmosphere and stirring continued for 1 h under 400 psi hydrogen atmosphere at rt. After releasing the pressure, triethylamine (4 mg, 38.8 μmol) was added. After stirring 30 min at rt, filtration and evaporation gave the crude triethylamine phosphate. In another flask, to the solution of uridine monophosphate **21** (7.6 mg, 15.0 μmol) in THF/DMF (3/1, 0.3 mL) was added carbonyl diimidazole (2.4 mg, 15.0 μmol). The stirring was continued for 2 h at rt, and the reaction was quenched by MeOH (0.01 mL). After 30 min, the reaction mixture was evaporated thoroughly, and transferred to the crude triethylammonium phosphate at rt. After 3 h, the reaction mixture was evaporated in *vaccuo* at 30 ^oC to give the crude coupling product. Aqueous NaOH (0.15 N, 0.26 mL) was added to the crude coupling product and the reaction mixture was stirred for 3h at rt. Purification by a sephadex-LH20 (0.05 M NH_4HCO_3/CH_3CN = 3/1) gave **3** (3.0 mg, 64%) as a white powder. Data for **3**: mp 252-253 ^oC; $[\alpha]^{20}_D$ +20.0 (*c* 0.15, H_2O); IR (KBr) 3312, 1749, 1701, 1632,

1212, 1162, 724 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 7.93 (d, *J* = 8.4 Hz, 1H), 5.97 (d, *J* = 4.0 Hz, 1H), 5.95 (d, *J* = 8.4 Hz, 1H), 5.47 (dd, J_1 = 3.2 Hz, J_2 = 7.2 Hz, 1H), 4.35 (m, 3H), 4.25 (m, 3H), 4.19 (m, 3H), 4.13 (m, 2H), 4.10-4.00 (m, 3H), 3.95 (m, 1H), 3.89-3.83 (m, 2H), 3.65 (t, *J* = 9.6 Hz, 1H), 2.89 (t, *J* = 7.6 Hz, 2H), 2.29 (t, *J* = 8.0 Hz, 2H), 2.12 (t, *J* = 7.2 Hz, 2H), 1.86 (m, 1H), 1.79 (m, 2H), 1.67 (m, 2H), 1.43 (m, 1H), 1.41 (d, *J* = 7.2 Hz, 3H), 1.39 (d, *J* = 6.4 Hz, 3H), 1.35 (d, *J* = 7.2 Hz, 3H), 1.32 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 179.98, 177.82, 175.99, 175.96, 175.18, 174.34, 174.25, 173.81, 166.44, 152.03, 141.82, 102.86, 94.66, 88.59 83.38 (d, *J* = 90.0 Hz), 79.68, 77.84, 73.92, 73.06, 69.84, 68.16, 65.18 (d, *J* = 67.5 Hz), 61.27, 60.44, 54.42, 54.35, 53.30, 51.16, 50.04, 49.81, 39.33, 31.96, 30.63, 28.25, 26.42, 22.19, 18.81, 17.62, 16.95, 16.63; ^{31}P NMR (121 MHz, D_2O) δ -10.64 (d, *J* = 20.7 Hz), -10.51 (d, *J* = 20.7 Hz); HRMS (ESI) Calcd. for $C_{40}H_{64}N_9O_{27}P_2$ $(M-H)^-$: 1164.3381; found: 1164.3392.

Synthesis of Uridine 5'-diphospho-MurNGlyc-L-Ala-γ-D-Glu(ONa)-L-Lys(FITC)-D-Ala-D-Ala-ONa, 5. To a stirred solution of **3** (1.6 mg, 1.32 μmol), in 0.1 M $NaHCO_3$ (0.10 mL), was added fluorescein isothiocyanate (0.8 mg, 1.98 μmol) in DMF (0.1 mL). After 2 h at rt, fluorescein isothiocyanate (0.8 mg, 1.98 μmol) in DMF (0.2 mL) and triethylamine (0.05 mL) was added. After stirring 12 h at rt, all volatiles were evaporation at below 30 °C to provide the crude product. Purification by a sephadex-LH20 chromatography (0.05 M NH_4HCO_3/MeCN = 3/1) gave the desired product **5** (2.0 mg, 95%) as an orange powder. Data for **5**: $[\alpha]^{20}_D$ +23.3 (*c* 0.2, H_2O); IR (KBr) 3310, 1750, 1701, 1633, 1168, cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 7.90 (d, *J* = 8.0 Hz, 1H), 7.68 (s, 1H), 7.55 (d, *J* = 7.2 Hz, 1H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.20-7.26 (m, 2H), 6.66 (m, 4H), 5.92 (m, 2H), 5.45 (dd, J_1 = 3.2 Hz, J_2 = 7.2 Hz, 1H), 4.34-4.03 (m, 14H), 3.84 (m, 2H), 3.63 (m, 2H), 2.27 (m, 2H), 2.10 (m, 2H), 1.89-1.80 (m, 4H), 1.66 (m, 2H), 1.39 (d, *J* = 7.6 Hz, 3H), 1.37 (d, *J* = 8.0 Hz, 3H), 1.35 (d, *J* = 7.6 Hz, 3H), 1.30 (d, *J* = 7.2 Hz, 3H); HRMS (ESI) Calcd. for $C_{61}H_{74}N_{10}O_{32}P_2S$ $(M-2H)^{2-}$: 776.1839; found: 776.1849.

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25. For the synthesis of **21**, see: Experimental Section.

26. Methyl 1*H*-imidazole-1-carboxylate is unreactive against alcohols, amines, and phosphate oxygens under neutral conditions at rt.

27. Similarly, BODIPY fluorophore could be conjugated to **3**.

28. A total synthesis of **4**. M. Kurosu and K. Li, unpublished data.