Acceptor Specificity and Inhibition of the Bacterial Cell-Wall Glycosyltransferase MurG

Haitian Liu, Thomas K. Ritter, Reiko Sadamoto, Pamela S. Sears, Min Wu, and Chi-Huey Wong*[a]

A continuous fluorescence coupled enzyme assay was developed to study the acceptor specificity of the glycosyltransferase MurG toward different lipid I analogues with various substituents replacing the undecaprenyl moiety. It was found that most lipid I analogues are accepted as substrates and, amongst these, the saturated C_{14} analogue exhibits the best activity. This substrate was used to evaluate the inhibition activity of such antibiotics as moenomycin, vancomycin, and two chlorobiphenyl vancomycin derivatives. A vancomycin derivative with a chlorobiphenyl moiety on the aglycon section was identified as a potent inhibitor of MurG.

KEYWORDS:

enzyme catalysis · glycosyltransferases · inhibitors · lipids · substrate specificity

Introduction

The bacterial cell-wall (peptidoglycan layer) provides strength and rigidity to the cell envelope, which allows bacteria to live in a hypotonic environment and gives them their characteristic shape. Antibiotic-induced defects lead to cell lysis as a result of the inability of the bacteria to cope with the internal osmotic pressure. Several natural products have been identified to inhibit different enzymes along the bacterial cell-wall biosynthesis pathway (Figure 1). For instance, moenomycin inhibits transglycosylase and vancomycin inhibits transpeptidase. This biosynthetic pathway thus provides an attractive target for antibiotics, as it has no counterpart in mammalian cells. However, bacteria have acquired resistance to most current antibiotics, including vancomycin, and this has seriously impacted our ability to combat infectious diseases.[1] To further understand the resistance mechanism at the molecular level and also to develop new antibiotics, we are studying the key events in peptidoglycan biosynthesis by using synthetic precursors and purified enzymes.

Bacterial cell-wall biosynthesis is a complex process that includes two glycopeptide lipid intermediates: undecaprenyl-pyrophosphyl-muramyl-pentapeptide (lipid I, **1**) and undecaprenyl-pyrophosphyl-muramyl-pentapeptide-GlcNAc (lipid II, **2**). Lipid I is formed from UDP-*N*-acetyl-muramyl-pentapeptide and undecaprenyl phosphate by MraY translocase; the reaction takes place on the cytoplasmic surface of the membrane. Lipid I then reacts with UDP-GlcNAc, in a process catalyzed by MurG, to form lipid II (Figure 1). Lipid II is subsequently polymerized and cross-linked into peptidoglycan by transglycosylation and transpeptidation.[2] We previously reported the successful chemoenzymatic synthesis of UDP-*N*-acetyl-muramyl-peptides, the substrates of MraY translocase, and the application of this method to the assembly of new bacterial architectures with a fluorescent probe or sugar analogue displayed on the surface.[3, 4] Now we have extended our interest into MurG, the last enzyme involved in the intracellular peptidoglycan biosynthesis. A sensitive coupled enzymatic assay for MurG has been developed to study the acceptor specificity of this enzyme, and the study has led to the identification of a simpler and better substrate that can be used to develop MurG inhibitors.

Results and Discussion

To set up an efficient enzymatic assay to evaluate potential inhibitors of MurG, we first need access to the natural substrate lipid I, which has a C_{55} undecaprenyl lipid chain with a *cis*-allylic pyrophosphate linkage to the sugar moiety. This long chain of lipid I together with the allylic pyrophosphate linkage complicates the synthesis, purification, and handling. Therefore, we wish to find an analogue of lipid I that is active for this enzyme and can be manipulated conveniently. Previous studies on this subject have been reported in a qualitative manner and several new substrates have been identified.[5, 6] We have further extended the study to evaluate different potential substrates and to determine their kinetic parameters. To determine whether the double bond in the lipid chain is necessary, we synthesized lipid I and the citronellol lipid I analogue **4** as well as other lipid I analogues with simple saturated alkyl chains. We also synthe-

[a] *Prof. C.-H. Wong, H. Liu, T. K. Ritter, Dr. R. Sadamoto, Dr. P. S. Sears, Dr. M. Wu*
Department of Chemistry and the Skaggs Institute for Chemical Biology
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-2409
E-mail: wong@scripps.edu

 DOI: 10.1002/cbic.200300557

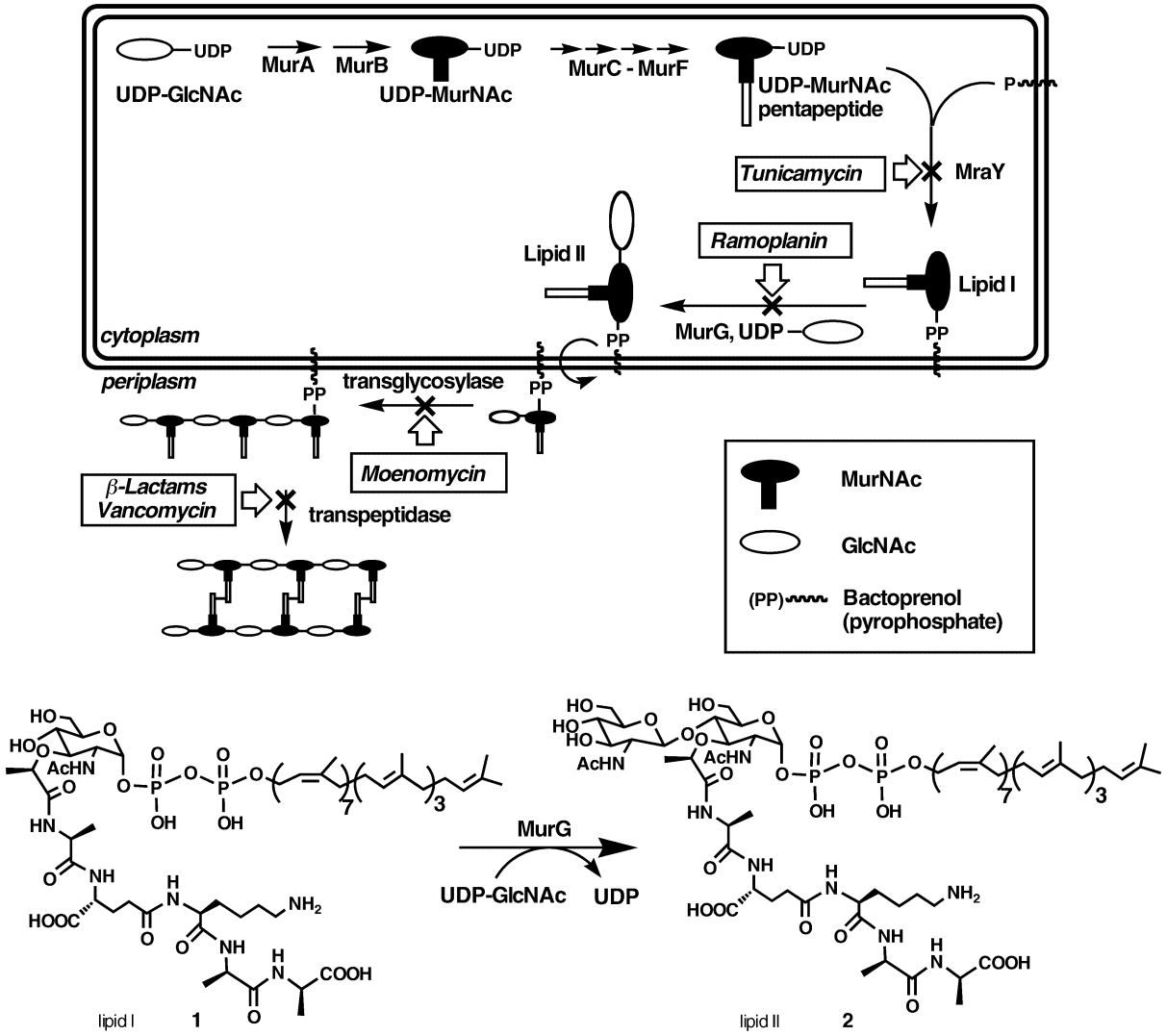
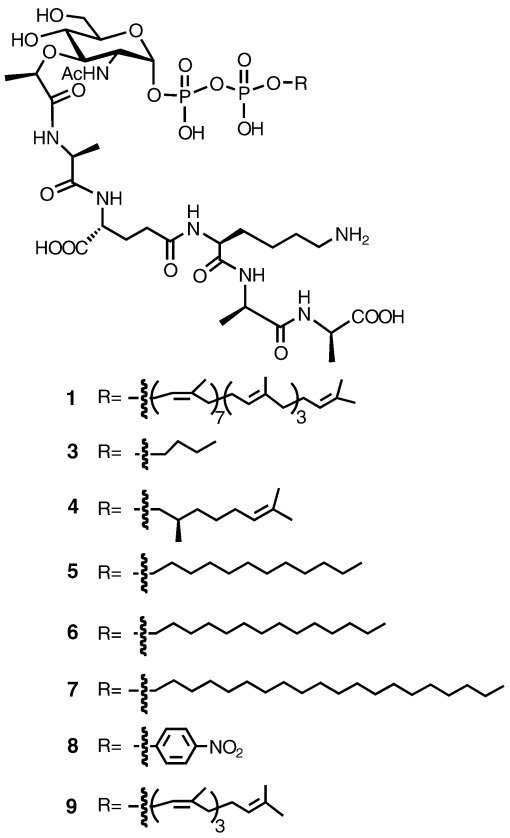


Figure 1. *Top: The pathway of bacterial cell-wall biosynthesis, highlighting the glycosyltransfer steps and their inhibitors. Bottom: Conversion of lipid I* ***1*** *into lipid II* ***2*** *catalyzed by MurG. GlcNAc = N-acetylglucosamine, UDP = uridine diphosphate.*

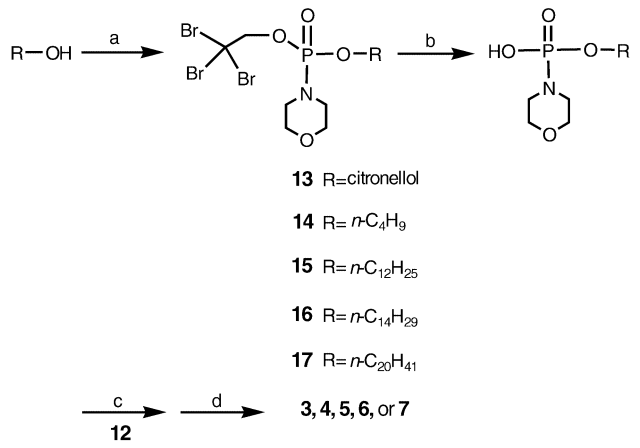
sized a nitrophenol lipid I analogue **8** to study whether a lipid chain is necessary at all (Scheme 1).

The first total synthesis of lipid I and the syntheses of several analogues containing lipid chains with varying lengths and double-bond geometries were recently reported.[5–7] We have prepared lipid I by using the published procedure. The preparation of lipid I nitrophenol analogue **8** is shown in Scheme 2. We coupled nitrophenol pyrophosphate derivative **11**, generated in situ by treating nitrophenol phosphate with diphenylchlorophosphate,[8] with sugar phosphate derivative **12**. After deprotection, the lipid I nitrophenol analogue **8** was obtained in nearly quantitative yield. The syntheses of other lipid I analogues are shown in Scheme 3. We treated different lipid alcohols with 2,2,2-tribromoethyl phosphoromorpholinochloridate to afford the corresponding phosphotriester derivatives (**13** – **17**) in 80 – 90 % yield. The phosphoromorpholidate derivatives were then obtained in high yields by selective removal of the 2,2,2-tribromoethyl protecting group with zinc dust. Coupling the phosphoromorpholidate derivatives directly with muramyl pentapeptide monophosphate (**12**) by using ^{1}H-tetrazole as a catalyst gave the desired pyrophosphate derivatives. Finally the lipid I analogues (**3** – **7**) were obtained following deprotection. It is noteworthy that the reported procedure for the synthesis of phosphoromorpholidates with 2,2,2-tribromoethyl phosphoromorpholinochloridate[9] has been adapted here to prepare the pyrophosphate derivatives from lipid alcohols, including the potentially unstable allylic pyrophosphate derivatives, with good reproducibility and high yield.[10] By using this method, a set of lipid I analogues containing lipid chains with varying lengths, including citronellol, C_4, C_{12}, C_{14}, and C_{20} alkyl chains, was conveniently prepared.

MurG was previously purified from *Escherichia coli* by using a cation-exchange step followed by size exclusion.[11] We added a His6 tag to the C terminus of MurG and purified the enzyme with Ni^{2+} affinity chromatography. This one-step purification yielded fully active MurG with greater than 95 % purity, shown as a single band around 38 kDa on a sodium dodecylsulfate PAGE gel.[12]

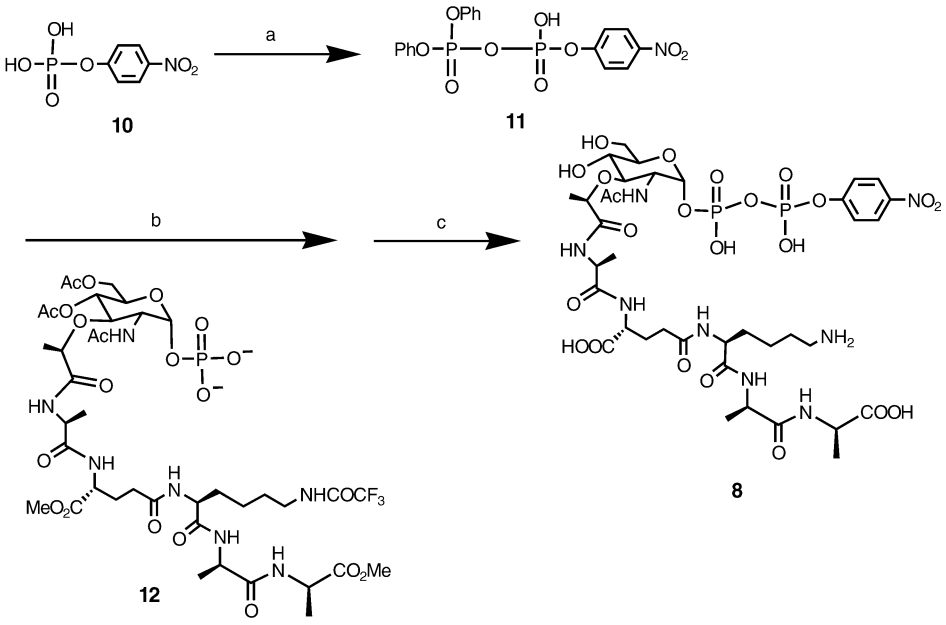


Scheme 1. *Structures of lipid I (**1**) and various lipid I analogues.*

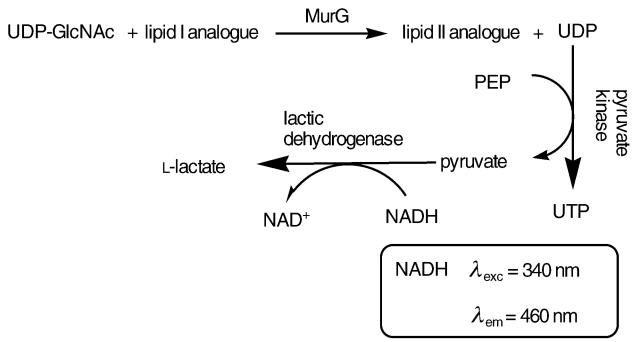


Scheme 3. *Reagents and conditions: a) 2,2,2-tribromoethyl phosphoromorpholinochloridate, pyridine, 80 – 90 %; b) Zn/tetrahydrofuran; c) ^{1}H-tetrazole, pyridine, **12**; d) NaOH, H_2O, 1,4-dioxane, 80 % (3 steps).*

A biotin-capture radioactive assay for MurG was developed for monitoring the transfer of radioisotopic carbon from UDP-[^{14}C]GlcNAc to the biotin-labeled acceptor analogue, which is captured by avidin affinity beads.[11] Recently, another radioactive assay that utilizes a dansylated lipid I analogue was reported.[13] End-point assays such as those are cumbersome and require radioisotopes. A continuous, nonradioactive assay would be desirable for the high-throughput analysis and kinetic study of this important enzyme in order to facilitate the discovery of new inhibitors. We have thus developed a continuous fluorescence coupled enzyme assay for monitoring MurG activity. In this assay, the formation of UDP is coupled to the decrease of NADH by using the modified pyruvate kinase – lactate dehydrogenase coupled assay of Burnell and Whatley (Scheme 4).[14] By monitoring the decrease of the fluorescence signal of NADH, MurG activity can be measured.[15] Using this continuous fluorescence coupled enzyme assay, we have determined the kinetic parameters of the lipid I analogues prepared above. The results are summarized in Table 1. MurG accepts all of the lipid I analogues as acceptors although there are big differences in specific activity (k_{cat}). The Micahaelis constant (K_M) values are similar, around 20 – 50 μM. Acceptor analogues **5** – **7** with saturated alkyl chains are better substrates than the natural lipid I and analogue **4**; this indicates that the double bond is not crucial for recognition. Interestingly, analogue **8** with the nitrophenol moiety replacing the lipid chain is a good substrate, which suggests that the interaction of the lipid chain with MurG is simply hydrophobic. On the other hand, there is a length requirement for the lipid chain. It should be long enough to interact hydrophobically with MurG, since analogue **3** with a C_4 alkyl chain is much less active than



Scheme 2. *Reagents and conditions: a) diphenylchlorophosphate, N,N-diisopropylethylamine, CH_2Cl_2; b) pyridine, CH_2Cl_2; c) NaOH, H_2O, quantitative (3 steps).*



Scheme 4. *Fluorescence coupled enzyme assay for MurG. NAD^+ = nicotinamide adenine dinucleotide, NADH = reduced nicotinamide adenine dinucleotide, PEP = phosphoenolpyruvate, UTP = uridine triphosphate.*

Table 1. *MurG acceptor specificity.*

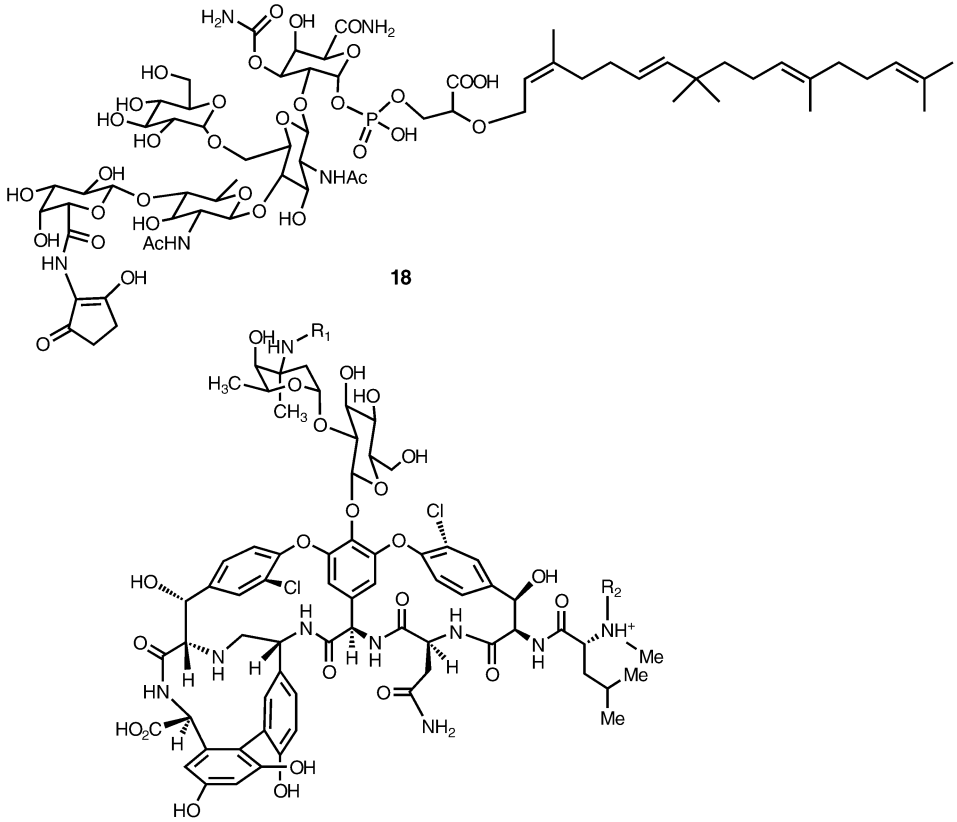
Acceptor/analogue	K_M [μM]	k_{cat} [min^{-1}]	k_{cat}/K_M [μM^{-1} min^{-1}]
3	22 ± 5	2 ± 0.1	0.09
1	20 ± 6	11 ± 2	0.55
4	47 ± 11	34 ± 5	0.72
4[a]	553 ± 125	134	0.24
8	24 ± 6	19 ± 3	0.79
7	52 ± 14	80 ± 9	1.54
5	29 ± 8	130 ± 16	4.40
6	39 ± 9	180 ± 13	4.62
9[a]	53 ± 6	837	15.8

[a] See ref. [16] for assay conditions.

analogues **4** – **7** with longer lipid chains. Among analogues **3**, **5**, and **6**, the reactivity increases as the chain length increases. Analogue **7** and the natural substrate lipid I are not as good as analogues **5** and **6**, probably due to solubility problems. Recently, a synthetic lipid I analogue **9** (Scheme 1) containing a tetraprenyl lipid chain was reported with $K_M = 53 \pm 6$ μM and $k_{cat} = 837$ min^{-1}.[15] We used the lipid I analogue **6** as an efficient substrate to test potential MurG inhibitors, because this analogue is more synthetically accessible and has the best activity among the analogues we prepared.

For development of novel antibiotics targeting MurG, we started the inhibitor test with several natural antibiotics including moenomycin (**18**) and vancomycin (**19**; Scheme 5). Both are inhibitors of the bacterial cell-wall biosynthesis pathway. Moenomycin, acting as a substrate analogue or possibly as a transition-state analogue, is believed to compete with lipid II for binding to the active site of transglycosylase.[16] Vancomycin, on the other hand, functions by binding to the C-terminal sequence D-Ala-D-Ala of lipid II, thereby impeding further processing of the monomeric unit into peptidoglycan.[17, 18] However, vancomycin and moenomycin cannot penetrate the bacterial cell membrane, so they don't encounter MurG in vivo because MurG localizes on the cytoplasmic side of the cell membrane.[19, 20] We reasoned that they probably would function as inhibitors of MurG in vitro since moenomycin might interfere with the enzyme and vancomycin would bind to the peptide portion of lipid I as well. We therefore used these two antibiotics to test the effectiveness of our new assay. Preliminary results showed that moenomycin and vancomycin did inhibit MurG at low micromolar concentrations.

Encouraged by these results, we further evaluated two chlorobiphenyl derivatives of vancomycin (Scheme 5). Previous studies have shown that a mono-*N*-aryl vancomycin derivative with the aryl group attached to the vancosamine amino moiety exhibits stronger antibacterial activity than the parent molecule and the *N*-aryl derivatives functionalized on the *N*-methyl leucine moiety.[21] In contrast, in the present MurG assay, we have found that derivative **21** with the chlorobiphenyl moiety substituted at



18

19 R^1 = H, R^2 = H
20 R^1 = *p*-chlorobiphenyl, R^2 = H
21 R^1 = H, R^2 = *p*-chlorobiphenyl

Scheme 5. *Structures of moenomycin (**18**), vancomycin (**19**), and two vancomycin derivatives.*

the *N*-methyl leucine residue is a better MurG inhibitor than vancomycin or derivative **20** where the chlorobiphenyl moiety is attached to the vancosamine group. These results suggest that, although both vancomycin and its derivatives inhibit the MurG-catalyzed reaction, they have other interactions with the substrate and/or the enzyme in addition to binding to the D-Ala-D-Ala moiety of the substrate. We have determined the IC_{50} values of the four compounds (Table 2). The vancomycin derivative **21** shows the best inhibition activity with an IC_{50} value of 1.25 μM. Compound **21** is greater than ten times as active as the parent vancomycin.

Table 2. *IC_{50} values of MurG inhibitors.*

Compounds	IC_{50} [μM]
18	10.6
19	15.7
20	21.4
21	1.25

The fact that moenomycin and vancomycin, two molecules with distinct structures, inhibit MurG implies that MurG interacts with some moiety in both molecules. Modification of vancomycin yielded a better inhibitor, derivative **21**, which is encouraging as optimization of inhibitors based on the framework of vancomycin is feasible. The search for inhibitors from moenomycin derivatives and the investigation of the inhibition mechanisms of both moenomycin and vancomycin derivatives are currently underway. This study also suggests that moenomycin and vancomycin derivatives may be found that can act as inhibitors of MurG and may enter the cell and function in vivo.

In summary, we have developed a sensitive coupled fluorescence assay for MurG to investigate its acceptor specificity and we have found an analogue with a C_{14} alkyl chain replacing the undecaprenyl group to act as an efficient substrate. We have also found that a vancomycin derivative with *N*-chlorobiphenyl-*N*-methyl leucine is a potent inhibitor of MurG. The MurG enzymatic assay with the alternative substrate should be useful for development of new inhibitors against this enzyme that may be potential antibiotics.

Experimental Section

Purification of *E. coli* MurG: The gene of MurG was cloned from *E. coli* genomic DNA and inserted into vector pET26(b) (Novagen). A His6 tag was added to the C terminus. After expression, the enzyme was purified by Ni^{2+} affinity chromatography according to the manufacturer's protocol (Qiagen).

Purification of moenomycin (18): A procedure has been developed for the purification of moenomycin from animal feed. Flavomycin complex (10 kg; Walco, CA) was extracted with MeOH/H_2O (1:5, 12 L) in a 50-liter carboy on an Innova 2000 platform shaker (65 rpm) for 22 days. The extract was decanted and the liquid was extracted with CH_2Cl_2. The aqueous layer was centrifuged at 8000 rpm for 20 min. The resulting clear brown solution was separated from the debris and applied to a column packed with Amberlite XAD-8 resin (20–60 μm, 30 × 740 mm, about 500 mL of sample was applied for each run). The column was washed with water (800 mL) and the moenomycin complex was eluted with methanol (1 L). The fractions containing the moenomycin complex were pooled, concentrated, and lyophilized. The moenomycin complex was further purified by flash column chromatography (silica gel; 2-propanol/2N NH_4OH (75:25)) to give the moenomycin C complex (C_1, C_3, and C_4; 0.5 g; $R_f = 0.45$, 2-propanol/2N NH_4OH (70:30)), moenomycin A (0.49 g, contaminated with moenomycin A_{12}, ratio of A:A_{12} = 7:1; HR MALDI MS: calcd for moenomycin A, $C_{69}H_{108}N_5O_{34}P$: 1580.6541 $[M-H]^-$; found: 1580.6626; $R_f = 0.39$, 2-propanol/2N NH_4OH (70:30)), and an unidentified compound which is not among the reported moenomycin family (0.28 g; ESI MS: 1487 $[M-H]^+$; $R_f = 0.33$, 2-propanol/2N NH_4OH (70:30)).

Synthesis of lipid I nitrophenol analogue 8: Nitrophenol phosphate bis(cyclohexylammonium) salt (100 mg, 0.23 mmol) was dissolved in CH_2Cl_2 (1 mL). *N*,*N*-diisopropylethylamine (0.1 mL) and diphenylchlorophosphate (0.07 mL, 0.35 mmol) were added to this solution at −10 °C, and the reaction mixture was warmed to room temperature and stirred for 2 h. The reaction was quenched with methanol (3 mL) and the solvents were evaporated. The residue (the crude nitrophenol diphosphate **11**) was coevaporated with dry toluene three times and dissolved in CH_2Cl_2 (1 mL). A portion of the solution (0.2 mL) was mixed with a solution of muramyl pentapeptide phosphate **12** (0.03 mmol) in CH_2Cl_2 (0.3 mL), and pyridine (40 μL) was added to the solution. The reaction mixture was stirred for 2 days and then the solvents were evaporated. Water (0.7 mL) and 2N NaOH (130 μL) were added and the mixture was stirred for 30 min. The aqueous solution was washed with diethyl ether once and loaded on a Bio-Gel P-4 column, which was eluted with 100 mM aq NH_4HCO_3. After lyophilization of the corresponding fraction, compound **8** (45 mg) was obtained as a white powder in nearly quantitative yield (calculated from compound **12**); ^1H NMR (D_2O, 600 MHz): $\delta = 8.22$ (d, $J = 8.76$ Hz, 2H), 7.34 (d, $J = 8.76$ Hz, 2H), 5.39 (m, 1H), 4.36–4.12 (m, 7H), 4.11 (m, 1H), 3.82 (m, 1H), 3.74 (m, 2H), 3.64 (t, $J = 9.18$ Hz, 1H), 3.54 (t, $J = 9.66$ Hz, 1H), 2.92 (t, $J = 7.02$ Hz, 2H), 2.27 (m, 2H), 2.11 (m, 1H), 2.00 (s, 3H), 1.72 (m, 2H), 1.61 (m, 2H), 1.36 (m, 14H) ppm; ES MS (negative): calcd for $C_{37}H_{58}N_8O_{23}P_2$: 1043 $[M-H]^-$; found: 1043.

Citronellol phosphotriester derivative 13: Citronellol (0.9 g, 5.8 mmol) was dissolved in anhydrous pyridine (3 mL), and 2,2,2-tribromoethyl phosphoromorpholinochloridate (2.9 g, 6.4 mmol) was added under Ar. After stirring for 24 h at room temperature, the mixture was diluted with CH_2Cl_2 (150 mL) and washed with brine twice. The organic layer was dried and concentrated in vacuo. Purification by flash column chromatography on silica gel eluting with CH_2Cl_2/EtOAc (3:1) yielded compound **13** (2.79 g, 85%) as a colorless syrup; $R_f = 0.5$ (CH_2Cl_2/EtOAc (3:1)); ^1H NMR ($CDCl_3$, 600 MHz): $\delta = 5.08$ (t, $J = 6.12$ Hz, 1H), 4.69–4.62 (m, 2H), 4.11 (m, 2H), 3.70 (m, 4H), 3.25 (m, 4H), 2.00 (m, 2H), 1.79 (m, 1H), 1.68 (s, 3H), 1.60 (s, 4H), 1.55 (m, 1H), 1.37 (m, 1H), 1.23 (m, 1H), 0.93 (dd, $J =$ 2.22 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 131.41$, 124.40, 78.81, 66.89, 66.85, 65.82, 65.78, 44.70, 37.58, 37.55, 37.35, 29.03, 25.70, 25.31, 19.30, 17.65 ppm; ES MS (positive): calcd for $C_{16}H_{29}Br_3NO_{24}P$: 568 $[M+H]^+$; found: 568.

Compound 14: Synthesized by a similar method to compound **13**; $R_f = 0.7$ (neat EtOAc); ^1H NMR ($CDCl_3$, 400 MHz) $\delta = 4.52$ (m, 2H), 3.98 (m, 2H), 3.59 (m, 4H), 3.12 (m, 4H), 1.59 (m, 2H), 1.32 (m, 2H), 0.83 (t, $J = 7.60$ Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz): $\delta = 78.67$, 67.05,

67.01, 66.74, 66.71, 44.56, 39.43, 32.50, 18.80, 13.66 ppm; ES MS (positive): calcd for $C_{10}H_{19}Br_3NO_4P$: 486 $[M+H]^+$; found: 486.

Compound 15: Synthesized by a similar method to compound **13**; $R_f = 0.3$ (hexanes/EtOAc (7:3)); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 4.52$ (m, 1H), 3.92 (m, 2H), 3.52 (m, 4H), 3.12 (m, 4H), 1.58 (m, 1H), 1.25 – 1.12 (m, 20H), 0.75 (t, $J = 6.20$ Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz): $\delta =$ 78.58, 67.25, 67.20, 66.65, 66.61, 44.48, 36.99, 31.66, 29.98, 29.38, 29.30, 29.25, 29.09, 28.88, 25.28, 22.44, 13.93 ppm; ES MS (positive): calcd for $C_{18}H_{35}Br_3NO_4P$: 598 $[M+H]^+$; found: 598.

Compound 16: Synthesized by a similar method to compound **13**; $R_f = 0.5$ (hexanes/EtOAc (7:3)); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 4.63$ (ddd, $J = 4.68$, 11.44, 24.36 Hz, 2H), 4.01 (m, 2H), 3.56 (m, 4H), 3.13 (m, 4H), 1.61 (m, 2H), 1.26 (m, 22H), 0.77 (t, $J = 6.48$ Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 78.68$, 67.31, 67.25, 66.75, 66.70, 44.58, 37.20, 31.85, 30.19, 29.62, 29.57, 29.49, 29.45, 29.28, 29.07, 25.46, 22.62, 14.00 ppm; ES MS (positive): calcd for $C_{20}H_{39}Br_3NO_4P$: 626 $[M+H]^+$; found: 626.

Compound 17: Synthesized by a similar method to compound **13**; $R_f = 0.3$ (hexanes/EtOAc (2:1)); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 4.70$ (ddd, $J = 3.52$, 11.44, 23.76 Hz, 2H), 4.32 (m, 2H), 3.69 (m, 4H), 3.24 (m, 4H), 1.73 (m, 2H), 1.25 (m, 34H), 0.89 (t, $J = 6.74$ Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz): $\delta = 78.79$, 67.46, 67.41, 66.87, 66.83, 44.67, 37.12, 31.87, 30.18, 29.64, 29.60, 29.51, 29.46, 29.31, 29.08, 25.46, 22.63, 14.08 ppm; ES MS (positive): calcd for $C_{26}H_{51}Br_3NO_4P$: 710 $[M+H]^+$; found: 710.

Compound 4: Citronellol phosphotriester derivative **13** (50 mg, 0.09 mmol) was dissolved in tetrahydrofuran (5 mL) and Zn powder (100 mg) was added in one portion. The suspension was stirred overnight. Zn dust was removed by celite filtration and the solution was concentrated in vacuo. The crude syrup was used for the next reaction without further purification.

Muramyl pentapeptide phosphate **12** (304 mg, 0.173 mmol) and the above compound (103 mg) in dry pyridine (4 mL) were coevaporated three times. ^1H-tetrazole (40 mg, 0.5 mmol) and anhydrous pyridine were added and the mixture was stirred for 2 days at room temperature. The reaction was terminated by adding water (200 μL) and the solvent was evaporated. The solid was dissolved in a mixed solution of 2N NaOH (1.6 mL) and water (3 mL) and stirred at room temperature for 1 h. The aqueous solution was washed with diethyl ether once and loaded on Bio-Gel P-4 column, which was eluted with 100 mM aq NH_4HCO_3. After lyophilization of the corresponding fraction, a white powder was obtained. The powder was dissolved in water (1 mL), filtered, and purified by reverse-phase HPLC on a Vydac 218TP C18 column (10 × 250 mm, flow rate = 4 mL min^{-1}, gradient elution of 100:0 to 30:70 A/B over 30 min where A = 0.05 M aq NH_4HCO_3 and B = MeCN). The retention time of the desired product was 15 min. Lyophilization of the column fractions afforded pure compound **4** (26 mg, 80%) as a white powder. Spectral analysis was in agreement with ref. [5].

Compound 3: Synthesized by a similar method to compound **4**; the compound was purified by reverse-phase HPLC on a Vydac 218TP C18 column (10 × 250 mm, flow rate = 4 mL min^{-1}, gradient elution of 100:0 to 30:70 A/B over 30 min where A = 0.05 M aq NH_4HCO_3 and B = MeCN). The retention time of the desired product was 5 min; ^1H NMR (D_2O, 600 MHz): $\delta = 5.37$ (m, 1H), 4.25 (m, 1H), 4.19 – 4.15 (m, 5H), 4.05 (m, 1H), 3.87 (m, 3H), 3.80 – 3.70 (m, 3H), 3.58 (t, $J = 9.66$, 9.18 Hz, 1H), 2.93(t, $J = 7.44$ Hz, 2H), 2.25 (t, $J = 7.44$, 7.50 Hz, 2H), 2.10 (m, 1H), 1.93 (s, 3H), 1.83 (m, 1H), 1.73 (m, 2H), 1.61 (m, 2H), 1.55 (m, 2H), 1.50 (m, 1H), 1.37(d, $J = 7.02$ Hz, 3H), 1.33 (d, $J = 7.02$ Hz, 3H), 1.29 (m, 9H), 0.83 (t, $J = 7.00$ Hz, 3H) ppm; HR FTMS (negative): calcd for $C_{35}H_{63}N_7O_{21}P_2$: 978.3479 $[M-H]^-$; found: 978.3441.

Compound 5: Synthesized by a similar method to compound **4**; the compound was purified by reverse-phase HPLC on a Vydac 208TP C8 column (4.6 × 250 mm; flow rate = 1 mL min^{-1}; gradient elution of 100:0 to 50:50 A/B over 50 min where A = 0.05 M aq NH_4HCO_3 and B = MeOH). The retention time of the desired product was 6 min; ^1H NMR (D_2O, 500 MHz): $\delta = 5.41$ (m, 1H), 4.30 (m, 1H), 4.23 – 4.14 (m, 7H), 4.11 (m, 2H), 3.90 (m, 3H), 3.84 – 3.76 (m, 3H), 3.60 (t, $J = 9.55$, 9.15 Hz, 1H), 2.97 (t, $J = 7.35$, 7.70 Hz, 2H), 2.27 (t, $J = 7.35$ Hz, 2H), 2.13 (m, 1H), 1.97 (s, 3H), 1.90 (m, 1H), 1.76 (m, 2H), 1.65 (m, 2H), 1.60 (m, 2H), 1.50 (m, 1H), 1.42(d, $J = 7.30$ Hz, 3H), 1.38 (d, $J = 6.95$ Hz, 3H), 1.34 (dd, $J = 2.60$, 2.20 Hz, 7H), 1.24 (m, 13H), 0.84 (t, $J = 7.44$ Hz, 3H) ppm; HR FTMS (negative): calcd for $C_{43}H_{79}N_7O_{21}P_2$: 1090.4731 $[M-H]^-$; found: 1090.4709.

Compound 6: Synthesized by a similar method to compound **4**; the compound was purified by reverse-phase HPLC on a Vydac 218TP C18 column (10 × 250 mm; flow rate = 4 mL min^{-1}; gradient elution of 100:0 to 20:80 A/B over 27 min and 20:80 to 0:100 A/B over 20 min where A = 0.05 M aq NH_4HCO_3 and B = MeCN). The retention time of the desired product was 5 min; ^1H NMR (D_2O, 600 MHz): $\delta = 5.31$ (m, 1H), 4.26 – 4.00 (m, 7H), 3.86 – 3.69 (m, 7H), 3.57 (t, $J = 9.24$ Hz, 1H), 2.92 (t, $J = 7.50$ Hz, 1H), 2.23 (t, $J = 7.44$ Hz, 2H), 2.09 (m, 1H), 1.92 (s, 3H), 1.82 – 1.67 (m, 3H), 1.61 – 1.54 (m, 4H), 1.24 (m, 36H), 0.78 (t, $J = 6.60$ Hz, 3H) ppm; HR FTMS (negative): calcd for $C_{45}H_{83}N_7O_{21}P_2$: 1118.5093 $[M-H]^-$; found: 1118.5044.

Compound 7: Synthesized by a similar method to compound **4**; the compound was purified by reverse-phase HPLC on a Vydac 208TP C8 column (4.6 × 250 mm; flow rate = 1 mL min^{-1}; gradient elution of 90:10 A/B for 10 min, 90:10 to 40:60 A/B over 20 min, and 40:60 to 0:100 A/B over 20 min where A = 0.05 M aq NH_4HCO_3 and B = MeOH). The retention time of the desired product was 24 min; ^1H NMR (D_2O, 500 MHz): $\delta = 5.51$ (dd, $J = 7.50$, 4.38 Hz, 1H), 4.32 – 4.22 (m, 6H), 4.15 (m, 1H), 3.97 (m, 3H), 3.87 (m, 2H), 3.53 (t, $J = 9.18$ Hz, 1H), 2.93 (m, 2H), 2.30 (m, 3H), 2.00 (s, 3H), 1.46 (d, $J = 7.44$ Hz, 3H), 1.42 (d, $J =$ 6.54 Hz, 3H), 1.39 (m, 9H), 1.23 (m, 31H), 0.90 (t, $J = 7.20$ Hz, 3H) ppm; HR FTMS (negative): calcd for $C_{51}H_{95}N_7O_{21}P_2$: 1202.5983 $[M-H]^-$; found: 1202.6066.

Fluorescence coupled enzyme assay: Reactions were carried out in a quartz cuvette in 200-μL volume, and the decrease in NADH fluorescence was monitored at 460 nm by using a Hitachi F-2000 spectrometer. Each reaction contained MurG reaction buffer (50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 7.9), 5 mM $MgCl_2$), 15 mM KCl, 0.4 mM phosphoenolpyruvate, 20 U mL^{-1} of lactate dehydrogenase, 20 U mL^{-1} of pyruvate kinase, 33 μM NADH, 167 μM UDP-GlcNAc, an appropriate amount of lipid I analogue, and enzyme (1 – 2 μL). All of the components except for NADH, the lipid I analogues, and MurG were premixed in an eppendorf tube and incubated at room temperature for 15 min. NADH and the substrate were added and the reaction mixture was incubated at 37 °C for 10 min. MurG was then added and the fluorescence was monitored.

IC_{50} value determination: For measurement of IC_{50} values, four different concentrations of inhibitors were tested at fixed concentrations of compound **6** (20 μM) and UDP-GlcNAc (167 μM). The rate data were plotted as reciprocal rate versus inhibitor concentration and the X-intercept is the $-IC_{50}$ value.

This research was supported by the National Institutes of Health (Grant: GM 44154). H.L. and T.K.R are predoctoral fellows of the Skaggs Institute for Chemical Biology. We thank Dr. Lac V. Lee and Dr. Thomas J. Tolbert for helpful discussions.

[1] H. C. Neu, *Science* **1992**, *257*, 1064 – 1073.
[2] J.-V. Höltje, *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 181 – 203.
[3] H. Liu, R. Sadamoto, P. S. Sears, C.-H. Wong, *J. Am. Chem. Soc.* **2001**, *123*, 9916 – 9917.
[4] R. Sadamoto, K. Niikura, P. S. Sears, H. Liu, C. H. Wong, A. Suksomcheep, F. Tomita, K. Monde, S. Nishimura, *J. Am. Chem. Soc.* **2002**, *124*, 9018 – 9019.
[5] H. Men, P. Park, M. Ge, S. Walker, *J. Am. Chem. Soc.* **1998**, *120*, 2484 – 2485.
[6] X.-Y. Ye, M.-C. Lo, L. Brunner, D. Walker, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2001**, *123*, 3155 – 3156.
[7] M. S. VanNieuwenhze, S. C. Mauldin, M. Zia-Ebrahimi, J. A. Aikins, L. C. Blaszczak, *J. Am. Chem. Soc.* **2001**, *123*, 6983 – 6988.
[8] C. D. Warren, R. W. Jeanloz, *Methods Enzymol.* **1978**, *50*, 122 – 137.
[9] J. H. van Boom, R. Crea, W. C. Luyten, A. B. Vink, *Tetrahedron Lett.* **1975**, *32*, 2779 – 2782.
[10] We used nerol as a model compound and found that the coupling reaction of the phosphoromorpholidate derivative with the α-anomeric phosphate group of *N*-acetylglucosamine-3,4,6-triacetate proceeded cleanly to give the desired product as judged by mass spectrometry.
[11] S. Ha, E. Chang, M.-C. Lo, H. Men, P. Park, M. Ge, S. Walker, *J. Am. Chem. Soc.* **1999**, *121*, 8415 – 8426.
[12] Crouvoisier et al. have reported purification of His6 tagged MurG to greater than 80%; see: M. Crouvoisier, D. Mengin-Lecreulx, J. van Heijenoort, *FEBS Lett*. **1999**, *449*, 289 – 292.
[13] G. Auger, J. van Heijenoort, D. Mengin-Lecreulx, D. Blanot, *FEMS Microbiol. Lett.* **2003**, *219*, 115 – 119.
[14] J. N. Burnell, F. R. Whatley, *Anal. Biochem.* **1975**, *68*, 281 – 288.
[15] During the preparation of this manuscript, a similar coupled fluorescence assay of MurG was reported; see: L. Chen, H. Men, S. Ha, X. Ye, L. Brunner, Y. Hu, S. Walker, *Biochemistry* **2002**, *41*, 6824 – 6833.
[16] M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J. M. Ghuysen, M. Nguyen-Disteche, *Mol. Microbiol.* **1999**, *34*, 350 – 364.
[17] H. R. Perkins, *Biochem. J.* **1969**, *111*, 195 – 205.
[18] J. C. Barna, D. H. Williams, *Annu. Rev. Microbiol.* **1984**, *38*, 339 – 357.
[19] N. El-Abadla, M. Lampilas, M. Hennig, P. Findeisen, P. Welzel, D. Muller, A. Markus, J. van Heijenoort, *Tetrahedron* **1999**, *55*, 699 – 722.
[20] C. Bordet, H. R. Perkins, *Biochem. J.* **1970**, *119*, 877 – 883.
[21] R. Nagarajan, *J. Antibiot.* **1993**, *46*, 1181 – 1195.

Received: January 13, 2003 [F 557]

