An Efficient Chemoenzymatic Strategy for the Synthesis of Wild-Type and Vancomycin-Resistant Bacterial Cell-Wall Precursors: UDP-*N*-acetylmuramyl-peptides

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The bacterial cell-wall biosynthesis pathway has been an attractive target for development of antibiotics; the penicillin and glycopeptide antibiotics such as vancomycin that inhibit the transpeptidation step are currently the most commonly used.[1] Recently, however, the emergence of bacterial resistance to most antibiotics, including vancomycin, threatens the effectiveness of these antibiotics.[2] To further understand the resistance mechanism and also to develop new antibiotics, we are designing inhibitors to target the polysaccharide backbone synthesis instead of the transpeptidation as no modification of the sugar moiety has been reported in the drug-resistant strains, and the enzyme catalyzing the polymerization of sugar building blocks, that is, transglycosylase, is fully exposed to the cell surface. A key element in this program is to prepare substrates and to develop a sensitive assay system. To this end, the wild-type cell-wall precursor UDP-*N*-acetylmuramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (UDP-*N*-MurNAc-pentapeptide **3**) and the vancomycin-resistant cell-wall precursor UDP-*N*-acetylmuramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Lac (UDP-*N*-MurNAc-depsipentapeptide **8**) have been prepared by both chemical and chemoenzymatic syntheses; and a sensitive assay for their incorporation into peptidoglycan has been developed to aid in the discovery of new antibiotics against vancomycin-resistant strains.

The biosynthesis of the peptidoglycan of bacterial cell-wall starts in cytosol and involves the synthesis of UDP-MurNAc-pentapeptide for wild-type bacteria or UDP-MurNAc-depsipentapeptide for vancomycin-resistant bacteria. The first total synthesis of UDP-MurNAc-pentapeptide was recently reported by Hitchcock et al.[3] We have found that using ^{1}H-tetrazole as a catalyst[4] greatly facilitated the coupling of muramyl pentapeptide and uridine monophosphomorpholidate (Scheme 1): The yield was significantly increased (nearly quantitative vs 32%), and the reaction time was reduced from two weeks to 2 days. Using a related strategy for the synthesis of a lipid I analogue,[5] we prepared UDP-MurNAc-depsipentapeptide involving the TEOC and TMSE protecting groups, which can be cleaved under mild conditions by TBAF to prevent the hydrolysis of the ester bond of D-lactate and D-alanine. As shown in Scheme 2, the synthesis commenced with the conversion of benzyl *N*-acetyl-4, 6-benzylidene muramic acid to anomeric dibenzyl phosphate in five steps, ready for appendage of the protected depsipentapeptide. Depsipentapeptide was first assembled by Mitsunobu reaction of commercially available L-lactate benzyl ester and Boc-D-Ala. We avoided using D-lactate and its derivative as starting materials because they are rather expensive and not optimal for large-scale synthesis. The remaining amino acids were added using standard Boc protection and EDC coupling procedures. Cleavage of the Boc group was carried out by evaporating at 60−65 °C with TsOH to keep the TEOC protecting group.[6] Coupling of the depsipentapeptide with the muramyl carboxyl fragment resulted in the corresponding amide **7**. Hydrogenolytic deprotection yielded the anomeric phosphate. The coupling of the phosphate with uridine 5′-monophosphomorpholidate using ^{1}H-tetrazole as a catalyst afforded the corresponding protected UDP-*N*-MurNAc depsipentapeptide. Finally, deprotection with TBAF yielded the desired product **8**. We noticed that treating with TBAF for more than 4 h resulted in the hydrolysis of the ester bond while using buffered TBAF resulted in decomposition.

An efficient chemoenzymatic strategy was then developed to synthesize compounds **3** and **8** (Scheme 3) starting with the enzymatic conversion of UDP-GluNAc to UDP-*N*-acetylmuramic acid via UDP-GluNAc enolpyruvate in one pot by MurA and MurB with in situ regeneration of NADPH from NADP based on glucose and a thermostable glucose dehydrogenase from *Bacillus sp*.[7] This one-pot procedure with regeneration of NADPH reduces the cost and eliminates the problem of product inhibition caused by NADP. Attaching the pentapeptide or the depsipentapeptide to UDP-*N*-acetylmuramic acid yielded the corresponding amides. After coupling, the deprotection route is different for the two compounds: For MurNAc-pentapeptide, deprotection with aqueous sodium hydroxide and purification by P-4 column gave the final product in 70% yield; For depsipentapeptide, the coupling product was deprotected by TBAF and purified by HPLC. Thus, the whole chemoenzymatic synthesis of the two cell-wall precursors took only three steps in 56% yield. Compared to the total chemical synthesis, the chemoenzymatic strategy is shorter, easier and less labor consuming. It avoids the cumbersome steps of protecting group manipulation. The preparative enzymatic synthesis of cytoplasmic intermediates of murein biosynthesis reported recently necessitates isolation and purification of all the enzymes in the pathway.[8] In addition, it is also difficult to make modifications to the peptide chain since the MurC-E enzymes usually only recognize their own substrates and do not tolerate changes well.[9] Compared with Scheme 2 and other published cell-wall substrate synthesis strategies,[3,5,7] the chemoenzymatic pathway reported here takes fewer steps and allows us to attach different peptide chains to UDP-*N*-acetylmuramic acid, making it possible to prepare analogues with different peptide chains in a short and easy way. As shown in Scheme 4, we prepared fluorescently labeled versions of **3**, in which the ϵ-amino group of the pentapeptide was reacted with NBD-F or with *N*-hydroxysuccinimide-activated fluorescein or rhodamine. The corresponding fluorescent products **11**, **12**, and **13** could also be incorporated into peptidoglycan.[10]

Incorporation of UDP-MurNAc-depsipentapeptide and UDP-MurNAc-pentapepide into peptidoglycan polymer in the absence of cross-linking was measured in a penicillin-insensitive assay with a particulate membrane fraction prepared from *Enterococcus faecalis* ATCC 29122.[11] In this assay, the membrane fraction catalyzes the transfer of UDP-MurNAc-pentapeptide or UDP-

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(9) MurC was tested to catalyze the attachment of L-Ala, L-Ala-D-Glu, L-Ala-L-Glu, L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala, and L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala respectively to UDP-MurNAc. We found MurC showed activity only for L-Ala, its original substrate.
(10) Incorporation of compounds **11**, **12**, and **13** into peptidoglycan was tested by radioactive assay using membrane fraction prepared from *E. faecalis* ATCC 29122.

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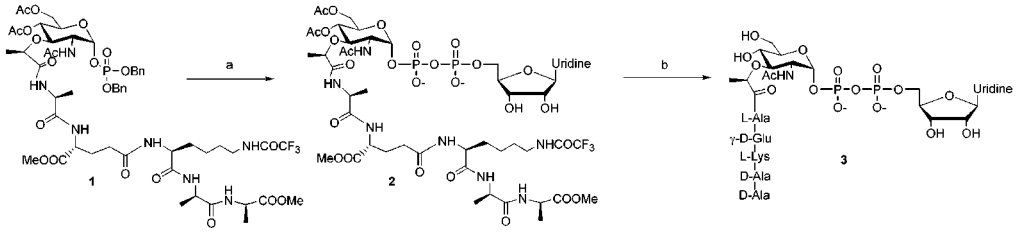
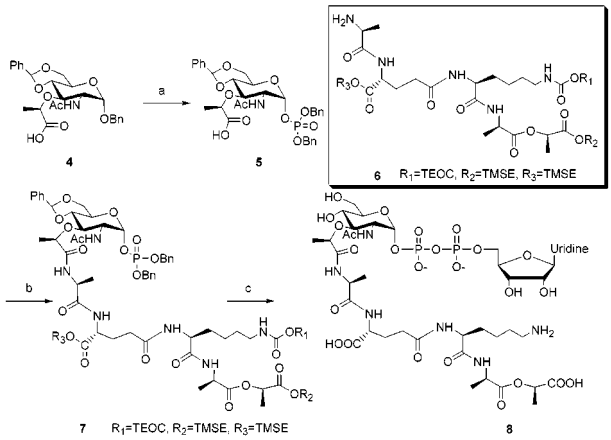
Scheme 1. Coupling of Muramyl Pentapeptide and Uridine Monophosphomorpholidate Catalyzed by ^{1}H-Tetrazole[a]



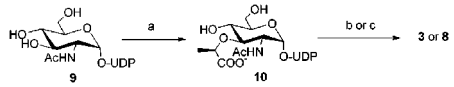
[a] (a) (i) H_2/Pd, CH_3OH; (ii) uridine 5′-monophosphmorpholidate, ^{1}H-tetrazole, pyridine; (b) NaOH aqueous, quantitative, three steps.

Scheme 2. Chemical Synthesis of UDP-MurNAc-depsipentapeptide[a]



[a] (a) (i) EDC, DMAP, 2-(phenylsulfonyl)ethanol, THF 95%; (ii) H_2/Pd, EtOAc; $PhCH(OCH_3)_2$, TsOH, DMF 78%, two steps; (iii) $^iPr_2NP(OBn)_2$, ^{1}H-tetrazole, CH_2Cl_2 −20 °C − 0 °C, then ^{t}BuOOH, −10 °C, 75%; (iv) DBU, CH_2Cl_2; (b) EDC, HOBt, DIEA, **6**, DMF 65%; (c) (i) H_2/Pd, CH_3OH; (ii) uridine 5′-monophosphmorpholidate, ^{1}H-tetrazole, pyridine; (iii) TBAF, DMF 50%, three steps.

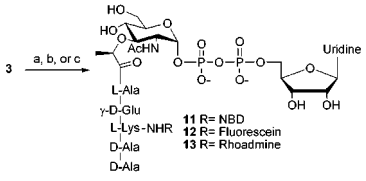
Scheme 3. Chemoenzymatic Synthesis of UDP-MurNAc-Peptides[a]



[a] (a) MurA, MurB, glucose dehydrogenase, PEP, glucose, NADP 80%; (b) (i) HBTU, DIEA, pentapeptide salt, DMF; (ii) NaOH aq 70% two steps; (c) (i) HBTU, DIEA, **6**, DMF; (ii) TBAF, DMF 65%, two steps.

MurNAc-depsipentapeptide to an endogenous lipid carrier undecaprenyl phosphate, followed by the addition of radioactively labeled UDP-GluNAc. The resulting disaccharide-peptide is subsequently polymerized to form the immature peptidoglycan in the presence of penicillin. Polymerization was measured with or without vancomycin. Both UDP-MurNAc-pentapeptide and UDP-MurNAc-depsipentapeptide were converted into polymerized peptidoglycan when there was no antibiotic added. On the other hand, when vancomycin was added, a dramatic decrease in the radioactivity of the peptidoglycan was observed in UDP-MurNAc-pentapeptide assay, indicating that vancomycin inhibits the incorporation of UDP-MurNAc-pentapeptide into peptidoglycan. In contrast, this dramatic decrease was not observed when the UDP-MurNAc-depsipentapeptide was used; suggesting that polymerization in the presence of depsipentapeptide precursors is resistant to vancomycin (Table 1).

Scheme 4. Preparation of Fluorescently Labeled UDP-MurNAc-Pentapeptide[a]



[a] (a) DIEA, NBD-F, THF, MEOH 60%; (b) NHS-fluorescein, PBS, 0 °C, then ethanolamine 50%; (c) NHS-rhodamine, PBS, 0 °C, then ethanolamine 10%.

Table 1. Effects of Vancomycin on the in Vitro Peptidoglycan Polymerization of UDP-MurNAc-pentapeptide and UDP-MurNAc-depsipentapeptide

precursor	antibiotic	radioactivity in peptidoglycan (dpm)	radioactivity in lipid-bound intermediate (dpm)
UDP-MurNAc-pentapeptide[a]	none	302	6195
	vancomycin[c]	152	2552
UDP-MurNAc-depsipentapeptide[b]	none	438	3591
	vancomycin[c]	435	3573

[a] Membrane added was 10 uL. [b] Membrane added was 20 uL. [c] Concentration used was 100 μg/mL.

In conclusion, we have prepared the bacterial cell-wall precursor UDP-MurNAc-pentapeptide and the vancomycin-resistant cell-wall precursor UDP-MurNAc-depsipentapeptide by chemical and chemoenzymatic syntheses. The chemoenzymatic route is simple and effective and provides flexibility to change the peptide portion. In addition, enzymatic assay of these synthesized cell-wall precursors has confirmed that the incorporation of UDP-MurNAc-depsipentapeptide into peptidoglycan is not inhibited by vancomycin, in contrast to that of UDP-MurNAc-pentapeptide. Together with other cell-wall synthesis pathway intermediates, the vancomycin-resistant bacterial cell-wall precursor and its wild-type precursor UDP-MurNAc-pentapeptide reported here are being used in our assay to identify new antibiotics.

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Supporting Information Available: Experimental details for the synthesis of the compounds **3**, **8**, **10**, **11**, **12**, and **13**; spectral data for compounds **6**, **7**, **8**, **11**, and Boc-D-Ala-D-Lac-OBzl (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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