Preparative Enzymatic Synthesis and Characterization of the Cytoplasmic Intermediates of Murein Biosynthesis

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Received November 6, 1998

Abstract: The first six cytoplasmic intermediates of murein biosynthesis (**2**−**7**) have been prepared in high yield (>60%) and at preparative scale (10−100 mg) using purified enzymes of the murein biosynthetic pathway (MurA−MurF). For at least three of these compounds, **5**, **6**, and **7**, this is the first high-yield synthesis and purification by any means that has been reported. Detailed spectroscopic and analytical data accompany each of the six compounds and serve as reference standards for future work. The availability of these intermediates will facilitate the study of the murein pathway enzymes MurA−MurF, all of which are validated antibacterial targets.

Introduction

Cell wall, or murein, biosynthesis is an essential and uniquely bacterial process and thus is an attractive target for antibiotic action. While many antibacterial compounds, such as β-lactams, vancomycin, fosfomycin, and bacitracin, target this complex pathway,[1] the emergence of bacterial resistance[2] now threatens the effectiveness of these (and virtually all other) antibiotics. The seriousness of the medical concern has invigorated the search both for antibacterial compounds of novel chemical structure as well as for new mechanisms by which to kill bacteria. The cytoplasmic steps of murein precursor assembly, catalyzed by the enzymes MurA[3] through MurF (Scheme 1), offer a wealth of unexploited targets for which permeable inhibitors might be found.

Since temperature-sensitive mutants of each of the *murA* through *murF* genes are lethal and each of the wild type genes are highly conserved among a wide range of bacteria, permeable inhibitors of any one of the *mur* gene products will be bacteriocidal and have a wide spectrum. Screening these enzymes for such compounds, either singly or combinatorially using a reconstituted murein pathway[4] of MurA through MurF (Scheme 1), requires pure pathway intermediates. Unfortunately, many of these intermediates are not readily available to support the necessary mechanistic and structural studies of these enzymes.[5] Here, we report a unified protocol for the rapid synthesis and purification of 10−100 mg quantities of murein substrates **2**−**7**. We also provide detailed characterization, including complete ^{1}H and ^{13}C NMR assignments, elemental analysis, and mass spectral data, for each of the intermediates, many of which heretofore have not been chemically characterized.

Materials and Methods

Materials. The disodium salt of UDP-*N*-acetyl glucosamine, phosphoenol pyruvate hexaammonium salt, β-NADH, β-NADPH, DTT, ATP, and ammonium acetate were purchased from Sigma. Pyruvate kinase type III from rabbit muscle, L-lactate dehydrogenase type XXIX-S from porcine muscle, L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanine-D-alanine were also obtained from Sigma. Ammonium acetate buffers were prepared by titrating with ammonium hydroxide or acetic acid to obtain the desired pH.

UDP-*N*-acetyl muramyl substrates were assayed routinely following the consumption of NADH (at 340 nm wavelength, $\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}) in a pyruvate kinase/lactate dehydrogenase-coupled assay to detect the enzymatic formation of ADP (converting PEP to pyruvate with regeneration of ATP). Reaction mixtures (400 μL) contained bis-Tris propane (100 mM), NADH (200 μM), ATP (1 mM), PEP (10 mM), $MgCl_2$ (5 mM), DTT (1 mM), pyruvate kinase (215 units), and L-lactate dehydrogenase (995 units). The amino acids L-alanine, D-glutamic acid, and *meso*-diaminopimelic acid were dissolved in water. The reaction is initiated by the addition of 5 μL of Mur enzymes (typical protein concentration of 2 mg/mL). The extents of reaction of substrates **2** and **3** were assayed by adding MurB, MurC, and L-alanine, whereas the extent of reactions of substrates **4**, **5**, **6**, and **7** were assayed by adding only reaction mixtures and assaying for ADP, since ADP is produced in the reactions.

^{1}H NMR spectra were recorded on either a Bruker AMX-500 (500 MHz) or a Varian Mercury-400 (400 MHz) spectrometer. Chemical shifts are reported in ppm from trimethylsilane with the solvent resonance as the internal standard ($CDCl_3$: δ 7.24 ppm, CD_3OD: δ 3.25 ppm, D_2O: δ 4.80 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, br = broad, m = multiplet), integration and coupling constants (Hz). ^{13}C NMR spectra were recorded on either a Bruker AMX-500 (125 MHz) or a Bruker DPX-300 (75 MHz) spectrometer. Chemical shifts are reported in ppm from trimethylsilane with the solvent resonance as the internal standard ($CDCl_3$: δ 77.0 ppm, CD_3OD: δ 49.8 ppm). Dioxane (δ 66.5 ppm) was added to D_2O as internal standard. By employing a combination of two-dimensional NMR

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(3) The capitalized gene name in roman type is used to refer to the gene product throughout (i.e., the gene product of *murA* is designated MurA).

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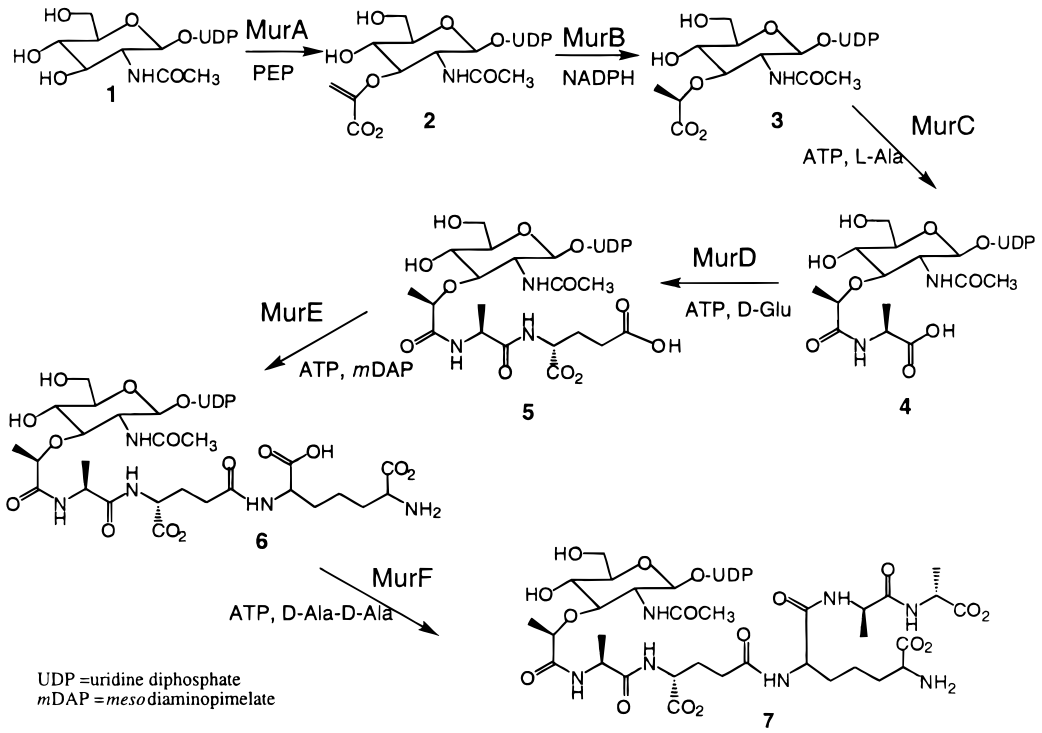
10.1021/ja983850b CCC: $18.00
Published on Web 01/27/1999

Scheme 1



techniques, including COSY (proton−proton correlation), HMQC (one-bond proton−carbon correlation), and HMBC (two- and three-bond proton−carbon correlation), it was possible to assign the proton and carbon spectra for each of the murein enzyme substrates with few ambiguities. In the proton spectrum, many resonances overlap, particularly in the region from δ 4.5 to δ 3.5, and peaks corresponding to individual protons are sometimes impossible to discern in the one-dimensional proton spectrum. In these cases, chemical shifts were assigned via cross-peaks in the HMQC spectrum. Occasionally, precise determination of chemical shift was impossible due to overlap of HMQC cross-peaks, and in these cases the chemical shifts for the overlapping protons are given as a range. Microanalytical data were obtained from Robertson Microlit Laboratories, Inc. NJ. Mass spectra were recorded on a Finnigan MAT TSQ700 triple quadropole mass spectrometer for electrospray ionization (ESI). HPLC analysis was performed using YMC AX300 weak anion-exchange column (4.6 mm internal diameter × 250 mm length) and run using an isocratic gradient of 300 mM KH_2PO4 pH 3.5 (1.5 mL/min over 25 min). The reaction mixtures were concentrated by overnight lyophilization with Virtis Benchtop 3L lyophilizer. All UV/vis spectra were recorded using a HP8425A diode-array UV/visible spectrophotometer (Hewlett-Packard) equipped with a thermostated, multicell-transport cell holder connected to a RM6 Lauda thermostat.

Synthesis of UDP-*N*-Acetylglucosamine Enolpyruvate (2). A reaction mixture containing TAPS (100 mM, pH 7.5), UDP−*N*-acetyl glucosamine (**1**) (32.5 mg, 50 mmol), and PEP (18.7 mg, 70 mmol) was prepared in a round-bottom flask. MurA (0.056 mg/mL) was added to the reaction mixture and incubated at 37 °C for 5 h. The extent of the reaction was determined by assaying for **2** with MurB and NADPH, following the decrease in absorbance at 340 nm. The reaction was complete within 5 h at 37 °C. The reaction mixture was filtered through an Amicon YM 10 membrane to remove the enzymes. Part of the reaction mixture (5 mL) was applied to a MonoQ anion-exchange column (10 × 10 cm, Pharmacia) and eluted with a nonlinear gradient from 0.02 to 1.0 M NH_4OAc, pH 8.0. The active fractions were pooled, concentrated, and lyophilized. The lyophilized flocculent powder was dissolved in water and lyophilized three times to remove the ammonium acetate buffer. The residue was dissolved in water and stored at −80 °C. Product **2** (30 mg, 92%) was analyzed by FAB mass spectrometry, HPLC, ^{1}H and ^{13}C NMR. MS (FD) *m*/*z* 679 (100%, M + H).

UDP-*N*-Acetylmuramic Acid (3). A reaction mixture containing TAPS (50 mM, pH 8.0), UDP-*N*-acetyl glucosamine (150 mg, 230 mmol), PEP (114 mg, 427 mmol), β-NADPH (237 mg, 284 mmol), and DTT (4 mmol) was prepared in a round-bottom flask and flushed with nitrogen for 30 min. MurA (0.056 mg/mL) and MurB (0.056 mg/mL) were added, which initiated the reaction, and the reaction flask was flushed with nitrogen for 10 min. The extent of the reaction was determined by assaying for **3** using MurC and the pyruvate kinase/lactate dehydrogenase-coupled assay. The reaction was complete within 5 h at 37 °C or overnight at 25 °C. The workup of the reaction was carried out as in the case of **2** except that the MonoQ anion-exchange column (10 × 10 cm Pharmacia) was eluted with a nonlinear gradient from 0.02 to 1.0 M NH_4OAc, pH 5.0. The active fractions were pooled, concentrated, and lyophilized. The lyophilized flocculent powder was dissolved in water and lyophilized three times to remove ammonium acetate. Finally, the residue was dissolved in water and stored at −80 °C. Product **3** (130 mg, 87%) was analyzed by FAB mass spectrometry, HPLC, ^{1}H and ^{13}C NMR, and elemental analysis. MS (FD) *m*/*z* 680 (100%, M + H); Anal. Calcd for formula $C_{20}H_{31}N_3O_{19}P_2$: C, 29.27; H, 6.14; N, 10.24; P, 7.55. Found: C, 29.35; H, 5.86; N, 10.56; P, 7.24.

UDP-*N*-Acetylmuramyl-L-alanine (4). Purified **3** was used as a starting material to synthesize **4**. The reaction mixture contained TAPS (50 mM, pH 8.0), ATP (475 mmol), $MgCl_2$ (190 mmol), DTT (95 mmol), L-alanine (380 mmol), and **3** (123.5 mmol). The MBP fusion MurC (0.2 mg/mL) was added to initiate the reaction. The extent of the reaction was followed by quantitating the amount of ADP present in the reaction mixture using the pyruvate kinase/lactate dehydrogenase-coupled assay. The reaction was complete within 5 h at 37 °C. The workup of the reaction was carried out as in the case of **2** except that the Mono Q anion-exchange column (10 × 10 cm, Pharmacia) was eluted with a nonlinear gradient from 0.02 to 1 M NH_4OAc, pH 9.0. The active fractions were pooled, concentrated, and lyophilized. The lyophilized material was dissolved in 20 mL of water, and 5 mL of it was applied to the pre-equilibrated MonoQ column with a nonlinear gradient of 0.02−1 M NH_4OAc, pH 5.0 buffer. Fractions containing the product were pooled and lyophilized. The lyophilized flocculent powder was dissolved in water and lyophilized three times to remove ammonium acetate. Finally, the residue was dissolved in water and stored at −80 °C. Product **4** (66 mg, 79%) was analyzed by FAB mass spectrometry, HPLC, ^{1}H and ^{13}C NMR, and elemental analysis. MS (FD) *m*/*z* 751.1 (60%, M + H); Anal. Calcd for $C_{23}H_{36}N_4O_{20}P_2$: C, 29.23; H, 6.29; N, 11.86; P, 6.55. Found: C, 29.55; H, 6.22; N, 12.18; P, 6.18.

UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamic Acid (5). Purified **3** was used as a starting material to synthesize **5**. The reaction mixture contained TAPS (50 mM, pH 8.0), ATP (600 mmol), $MgCl_2$ (240 mmol), DTT (120 mmol), L-alanine (480 mmol), D-glutamic acid (600 mmol), and **3** (120 mmol). MBP fusion MurC (0.2 mg/ml) and MurD (0.25 mg/ml) were added to initiate the reaction. The progress of the reaction at 37 °C was followed by assaying for ADP activity as in the case of **4**. The reaction was complete within 5 h. The workup of the reaction was carried out as in the case of **2** except that the MonoQ anion-exchange column (10 × 10 cm, Pharmacia) was eluted with a nonlinear gradient from 0.02 to 1.0 M NH_4OAc, pH 9.0. The active fractions were pooled concentrated and lyophilized. The lyophilized material was dissolved in 20 mL of water and 5 mL of it was applied to the preequilibrated MonoQ column with a nonlinear gradient of 0.02−1M NH_4OAc pH 9.0 buffers. Fractions containing the product were pooled and lyophilized. The lyophilized flocculent powder was dissolved in water and lyophilized three times to remove ammonium acetate. Finally, the residue was dissolved in water and stored at −80 °C. Product **5** (60 mg, 74%) was analyzed by FAB mass spectrometry, HPLC, ^1H and ^{13}C NMR, and elemental analysis. MS (FD) *m*/*z* 880.2 (100%, M + H); Anal. Calcd for $C_{28}H_{43}N_5O_{23}P_2$: C, 29.38; H, 6.43; N, 13.46; P, 5.41. Found: C, 29.38; H, 6.36; N, 13.05; P, 5.13.

UDP-*N*-Acetylmuramyl-L-alanyl-γ-D-glutamyl-*m*-diaminopimelic Acid (6). To synthesize **6**, purified **3** was used as a starting material. The reaction mixture contained TAPS (50 mM, pH 8.0), ATP (225 mmol), $MgCl_2$ (90 mmol), DTT (45 mmol), L-alanine (135 mmol), D-glutamic acid (135 mmol), *meso*-diaminopimelic acid (135 mmol), and **3** (22.5 mmol). MBP fusion MurC (0.2 mg/mL), MurD (0.25 mg /mL), and MurE (0.25 mg/mL) were added to initiate the reaction. The progress of the reaction was followed by assaying for ADP activity as in the case of **4** and **5**. The reaction was complete within 5 h at 37 °C. The work up of the reaction was carried out as in case of **2** except MonoQ anion-exchange column (10 × 10 cm Pharmacia) was eluted with a nonlinear gradient from 0.02 to 1 M NH_4OAc, pH 5.0. The active fractions were pooled, concentrated and lyophilized. The lyophilized material was dissolved in 20 mL of water, and 5 mL of it was applied to the preequilibrated MonoQ column with a nonlinear gradient of 0.02−1.0 M NH_4OAc, pH 9.0 buffers. Fractions containing product were pooled and lyophilized. The lyophilized flocculent powder was dissolved in water and lyophilized three times to remove ammonium acetate. Finally, the residue was dissolved in water and stored at −80 °C. Product **6** (10.4 mg, 68%) was analyzed by FAB mass spectrometry, HPLC, and ^1H and ^{13}C NMR. MS (FD) *m*/*z* 1052.9 (100%, M + H). Anal. Calcd for $C_{35}H_{54}N_7O_{26}P_2$: C, 34.29; H, 6.49; N, 13.71; P, 5.05. Found: C, 34.70; H, 6.29; N, 13.55; P, 4.65.

UDP-*N*-Acetylmuramyl-L-alanyl-γ-D-glutamyl-*m*-diaminopimelyl-D-alanyl-D-alanine (7). To synthesize **7**, purified **3** was used as a starting material. The reaction mixture contained TAPS (50 mM, pH 8.0), ATP (175 mmol), $MgCl_2$ (70 mmol), DTT (35 mmol), L-alanine (105 mmol), D-glutamic acid (105 mmol), *meso*-diaminopimelic acid (105 mmol), D-alanine-D-alanine (105 mmol), and **3** (17.5 mmol). MBP fusion MurC (0.2 mg/mL), MurD (0.25 mg/mL), MurE (0.25 mg/mL) and MurF (0.25 mg/mL), were added to initiate the reaction. The progress of the reaction was followed by assaying for ADP activity as in the case of **4**, **5**, and **6**. The reaction was complete within 5 h at 37 °C. The workup of the reaction was carried out as in the case of **2** except that the MonoQ anion-exchange column (10 × 10 cm, Pharmacia) was eluted with a nonlinear gradient from 0.02 to 1 M NH_4OAc, pH 9.0. The active fractions were pooled, concentrated, and lyophilized. The lyophilized material was dissolved in 20 mL of water, and 5 mL of it was appilied to the pre-equilibrated MonoQ column with a nonlinear gradient of 0.02−1.0 M NH_4OAc, pH 5.0 buffers. Fractions containing the product were pooled and lyophilized. The lyophilized flocculent powder was dissolved in water and lyophilized three times to remove ammonium acetate. Finally, the residue was dissolved in water and stored at −80 °C. Product **7** (9.3 mg, 61%) was analyzed by FAB mass spectrometry, HPLC, and ^1H and ^{13}C NMR. MS (FD) *m*/*z* 1194.4 (100%, M + H). Anal. Calcd for $C_{41}H_{64}N_9O_{28}P_2$: C, 34.65; H, 6.81; N, 14.78; P, 4.36. Found: C, 34.81; H, 6.46; N, 14.52; P, 3.90.

Results and Discussion

Although cell wall precursors **3**−**7** will accumulate in bacteria treated with specific inhibitors of the murein pathway, purification of these compounds is inefficient and labor intensive.[6] Chemical synthesis is not an easy route either, given the stereochemical complexity of these intermediates, and only **1** and (the lysyl-containing version[7] of) **7** have been prepared synthetically.[8] Although enzymatic syntheses and purifications of **2**, **3**, and **4** have been reported, the HPLC purification protocol limited the amount of material that could be processed.[9] A larger scale, open-column chromatography method[10] to purify **3** using triethylamine ammonium bicarbonate (TEAB) as solvent produced, in our hands, a persistent contaminant of 317 amu (as revealed by FAB mass spectral analysis).[11] No high yield synthesis and purification of **5**, **6**, or **7** by any method has been reported.

The drawbacks of literature protocols compelled us to devise a generalized procedure for producing large quantities of *each* cell wall precursor. Since recombinant *Escherichia coli mur* genes can be overexpressed in soluble form in *E. coli* and purified in gram quantities,[12] enzymatic synthesis appeared to be the most efficient way to proceed. In short, the target compound was prepared by incubating **1** with the Mur enzyme(s) and their cosubstrates that preceded the target compound in the metabolic sequence. Each intermediate could be produced in turn, capitalizing on the in situ synthesis of the prior intermediate. Thus, synthesis of **2** from **1**[13] was straightforward using MurA (Table 1). Developing a one-pot, two-enzyme (MurA and MurB) synthesis of **3** from **1** to bypass the purification of **2** was complicated by the sensitivity of MurB to substrate and product inhibition,[9d] which precluded the use of high substrate concentration in the reaction. Using high concentrations of MurB mitigated the substrate inhibition problem but also revealed an intrinsic NADPH oxidase activity

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(7) Within eubacteria, the third position of the pentapeptide side chain of **7** is the most variable. There is no easy formula to predict which amino acid residue occurs in this position for a given bacterial species. Of the more than nine different amino acid residues known to occupy this position, the most common is *meso*-diaminopimelate (as in **7**), which is probably present in all Gram-negative bacteria. L-Lysine occurs frequently in the MurNAc-pentapeptide of Gram-positive cocci. For a review, see: Schleifer, K. H.; Kandler, O. *Bacteriol. Rev.* **1972**, *36*, 407−477.

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(11) Commercially available TEAB buffer and triethylamine base both contained a contaminant of the same mass. We suspect that this contaminant is a product of light-activated polymerization of triethylamine that yields a nonvolatile compound. Because of this problem, the TEAB solvent system was not used to purify the murein substrates.

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(13) **1** is commercially available or it can be prepared: ref 8a or Leiting, B.; Pryor, K. D.; Eveland, S. S.; Anderson, M. S. *Anal. Biochem.* **1998**, *256*, 185−191.

Table 1

substrate	scale (mg)	yield (%)
2	30	92
3	130	87
4	66	79
5	60	74
6	10.4	68
7	9.3	61

of MurB (constituting 10% of the normal reaction rate). Because of this side reaction, high levels of NADPH would be required, adding difficulty to the purification problem. However, by performing the reaction under argon, conversion of **1** to **3** was accomplished in >95% yield on 100 mg scale (Table 1).

Murein ligases MurC through MurF each catalyze an ATP-dependent addition of an amino acid to the free carboxylate of its substrate (Scheme 1). To synthesize **4**−**7**, we used **3** instead of **1** as the starting material to reduce the number and quantity of the reaction byproducts that need to be eliminated during the purification. Typical reactions included **3** (0.5−1.5 mM), the relevant murein ligases, and a high concentration of ATP (5 mM) to provide enough energy equivalence for the ligases as well as to drive the reaction to completion (Table 1). A potential worry is the susceptibility of MurD and MurF to substrate inhibition by high concentrations of UDP-*N*-acetylmuramyl-peptide (MurNAc-peptide) intermediates.[14] This concern can be overcome by running the reaction under conditions where the flux through each biosynthetic enzyme is roughly equal, allowing no buildup of any intermediate.[4]

Although running reactions is easy using enzymes, purifying the water-soluble target product from a reaction mixture containing closely related, highly charged reactants and byproducts is a real challenge. In general, after removing the enzymes by ultrafiltration and lyophilizing the reaction mixture, the desired murein intermediate was purified by FPLC anion-exchange chromatography (MonoQ) using an ammonium acetate solvent buffered at various hydrogen ion concentrations (pH 5.0, 8.0, or 9.0). All of the murein intermediates were purified varying only the pH in this chromatography system.

Compounds **2** and **3** were purified using ammonium acetate solvent at pH 8.0 and 5.0, respectively. For the purification of **4** and **5**, and especially of **6** and **7**, good separation from ADP and ATP is crucial because, as the number of ligase steps performed in one pot increases, so does the amount of ADP byproduct in the final mixture. Resolving MurNAc-peptide products **4**, **5**, **6**, or **7** from ATP and ADP required two passages through the anion-exchange column, the first time using ammonium acetate at pH 9.0 and then at pH 5.0, or vice versa. After solvent was removed from fractions containing target compound, the solid residue was dissolved in water and stored in aliquots at −80 °C. The murein intermediates are stable for at least one year.

Since few murein substrates have been spectroscopically characterized, each of the purified precursors was subjected to a battery of analytical inspections. Using ^{1}H and ^{13}C NMR, HPLC, FAB mass spectrometry, and elemental analysis, the murein substrates were all in the range of 96−99% pure (Figure 1). Comparison of the concentration derived from UV spectroscopic measurement (using $\epsilon_{262} = 10\ 100\ M^{-1}\ cm^{-1}$) with that derived from an enzymatic end-point assay gave excellent agreement. A combination of two-dimensional NMR techniques including proton−proton correlation (COSY), one-bond proton−carbon correlation (HMQC), and two- and three-bond proton−carbon correlation (HMBC) allowed for the complete proton and carbon peak assignments for **2**−**7** (see Supporting Information).

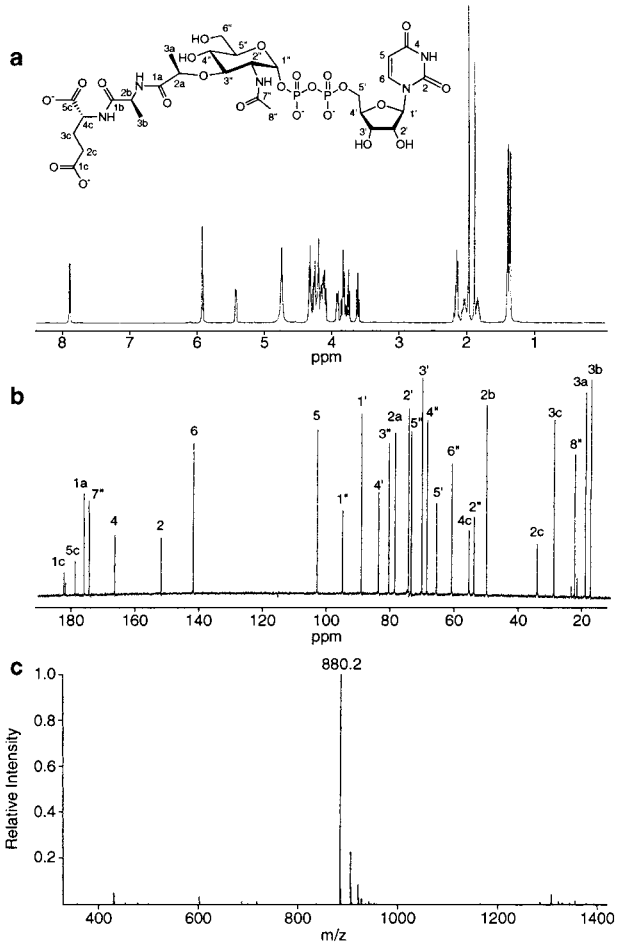

Figure 1. Representative spectroscopic data for murein pathway intermediates. (A) ^{1}H NMR spectrum of **5**, (B) ^{13}C NMR spectrum of **5** (C) FAB mass spectrum of **5**.

Other than a recent description of the Park nucleotide,[8b] this paper is the first to describe the detailed characterization of six murein pathway intermediates (**2**−**7**). The rapid synthesis (from commercially available **1**) and purification of these cell wall precursors will facilitate the study of the early steps of murein biosynthesis, a known target for antibiotic action. In addition, radio-labeled cell wall intermediates are easily prepared by including the relevant radio-labeled amino acid in the reaction mixture. More importantly, the availability of all murein substrates should allow for rapid analysis of inhibitors from murein pathway screens, to identify their enzyme target and elucidate their mechanisms of action.

Acknowledgment. The authors thank Barbara Leiting (MRL) for the recombinant stage I enzymes used for the synthesis of all the murein substrates, Tracey D. Klatt (MRL) for assistance in the mass spectral analysis, Renee M. Chabin (MRL) for HPLC analysis, and John W. Kozarich (MRL) for his unbridled encouragement and resolute support.

Supporting Information Available: Analytical and spectral data for all murein substrates, including complete proton and carbon NMR assignments, are provided (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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