Engineering a Cell-Free Murein Biosynthetic Pathway: Combinatorial Enzymology in Drug Discovery

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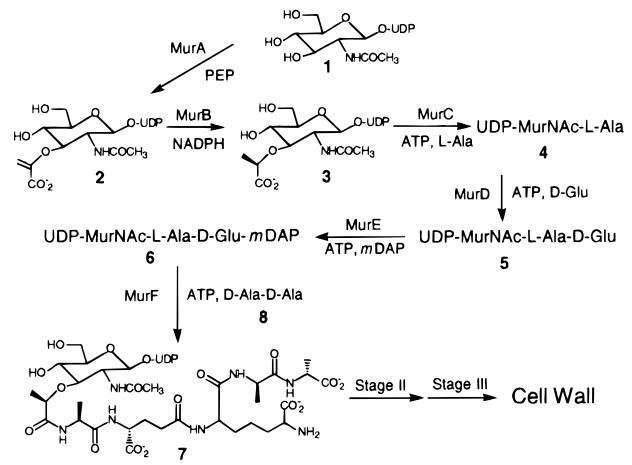
More than 40 antibiotics in clinical use, mostly β-lactams and glycopeptides, act by inhibiting the action of penicillin-binding proteins (PBPs) and attest to the usefulness of drugs that block the periplasmic stages of cell wall, or murein, biosynthesis. However, the increased prevalence and severity of resistance to these (and other) agents demand new approaches to antibiotic development. One group of antibacterial targets that has not been thoroughly examined for inhibitors—probably because their substrates are not easily isolated or synthesized—are the enzymes that catalyze the early cytoplasmic steps of cell wall biosynthesis, MurA−MurF[1] (Scheme 1). We have developed a novel and efficient pathway assay to screen simultaneously for inhibitors of the six enzymes in this metabolic sequence.

The cell wall is a single polymeric molecule consisting of glycan chains cross-linked with short unusual peptide bridges that maintains cell integrity against an osmotic pressure differential of over 4 atm.[2] Assembly of this uniquely bacterial structure begins in the cytoplasm (stage I) with the condensation of phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (**1**) catalyzed by MurA (Scheme 1). After a stereospecific reduction of **2** to **3** mediated by MurB, a series of ATP-dependent amino acid ligases (MurC, MurD, MurE, and MurF) catalyze the stepwise synthesis of the pentapeptide side chain using the newly synthesized lactoyl carboxylate as the first acceptor site to produce **7**. Attachment of **7** to an undecaprenyl phosphate carrier in the plasma membrane (stage II) via a diphosphate linkage is accompanied by release of UMP. Addition of another glucosamine unit to the 4-OH of the muramic acid moiety completes the synthesis of the monomeric building block. The finished precursor is moved across the membrane into the periplasm where it is stitched enzymatically by the PBPs into the fabric of the growing cell wall (stage III). All of the murein biosynthetic genes are essential; conditional lethal mutations are lytic.[3] Thus, chemical disruption of the enzymes they encode should be lethal.

Because all of the murein biosynthetic enzymes are valid targets, it is impossible to select a priori the "best" single target to screen for inhibitors. Thus, we reconstituted the murein pathway in vitro to assay simultaneously six of the enzymes in the biosynthetic sequence (MurA−MurF). Since the product of one enzyme is the substrate for the next enzyme in the pathway, the effort of preparing substrate for each individual enzyme is obviated. Only the first substrate (**1**) needs to be supplied. The cosubstrates of the pathway (PEP, ATP, NADPH, amino acids) are all common biochemicals. In fact, the in situ synthesis of substrates for enzymes that have rare substrates (i.e., not commercially available) makes screening for these enzymes possible.

Scheme 1



The *Escherichia coli mur* genes were expressed in soluble form in *E. coli* and purified in gram quantities.[4] Since the goal is to discover inhibitors of the pathway enzymes, and not to recreate a test tube version of the in vivo murein pathway, concentrations of the enzymes and substrates were optimized empirically so that an inhibitor of any one of the enzymes in the pathway is equally likely to be detected. In practice, this meant that the fluxes of substrate through each enzyme were equal and that the concentrations of substrates (except for cosubstrates PEP and ATP) were subsaturating.[5]

Using radiolabeled **1**, the rates of formation and decomposition of the intermediates of the pathway assay were quantitated using HPLC analysis (Figure 1).[6] Notably, conversion of **1** to **7** was not complete (75% yield) which is likely due to feedback inhibition of MurA by **6** and **7**.[7] When a potent inhibitor of *E. coli* MurD[8] (L-805,170) was included in the pathway assay, it blocked the formation of **7** in a concentration-dependent

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(1) 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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(5) With initial concentrations for all of the enzymes set to 50 nM and the concentrations of the substrates (except for ATP and PEP) set at their approximate K_M values, the rate of product formation was determined. The enzyme concentration of the first enzyme was reduced incrementally, keeping the other enzyme concentrations fixed, until the rate of product formation began to decrease linearly with the decrease in the varied enzyme concentration. This concentration of the first enzyme was then fixed. The same experiment was performed for subsequent enzymes in the pathway until the concentrations for all of the enzymes were adjusted to values where the flux of the pathway was equally determined by each of the constituent enzymes. Although we developed a computer simulation of the pathway kinetics, the individual kinetic parameters of the murein enzymes are not known completely, precluding a mathematical solution for the appropriate concentrations of enzymes to use in the assay.[13]

(6) The pathway assay contained in a final volume of 50 μL: bis-tris Propane (50 mM, pH 8.0), L-alanine, D-glutamate, *meso*-diaminopimelate (100 μM each); D-alanyl-D-alanine (10 μM), UDP-*N*-acetyl-glucosamine (12 μM), PEP (12 μM), NADPH (25 μM), DTT (500 μM), $(NH_4)_2SO_4$ (25 mM), KCl (5 mM), $MgCl_2$ (5 mM), ATP (500 μM), MurA, MurB, MurC, MurD, MurE, and MurF at final concentrations of 29.5 nM, 3.3 nM, 35.8 nM, 0.9 nM, 2.3 nM, and 2.1 nM, respectively and 10% DMSO. Inhibitors (dissolved in DMSO) were added to the desired concentrations. After this mixture in a volume of 45 μL was prepared, the reaction was initiated by adding [^{3}H]-**1** (5 μL of a 120 μM solution, 500 nCi) and then quenched after a timed interval by adding 200 μL of KH_2PO_4 (300 mM, pH 3.5). Samples in a 96-well plate were analyzed by anion-exchange HPLC using a 45-mL linear gradient of 90−195 mM KH_2PO_4, pH 3.5, and inline radioflow detection. Retention times of all of the intermediates of the pathway were determined by using authentic compounds.

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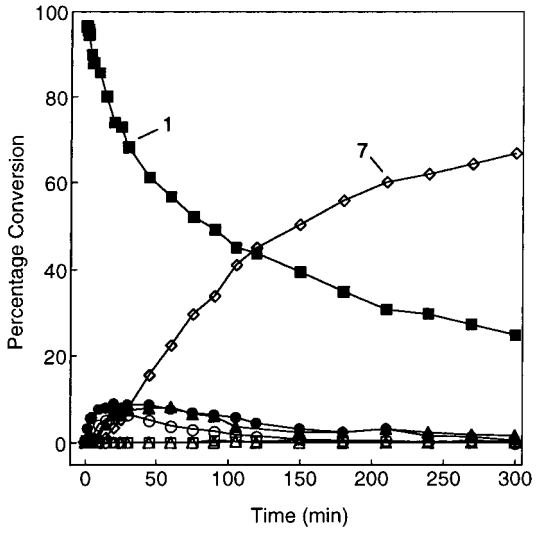

Figure 1. The murein pathway assay in action. Percent conversion of [^{3}H]-**1** (■) to pathway intermediates as a function of time. The formation of the final product (**7**, (◇)) has a lag phase of 20 min, followed by a linear rate which ultimately leveled off at about 75% conversion. Note that **3** (●), **4** (○), and **5** (▲) rise in concentration transiently before returning to a low steady-state concentration. Accumulation of **2** (□) and **6** (△) were not observed on the time scale of the experiment. The kinetics are consistent with a sequential built up of each intermediate to a concentration sufficient to support the next catalyzed reaction in the pathway. See ref 6 for reaction conditions.

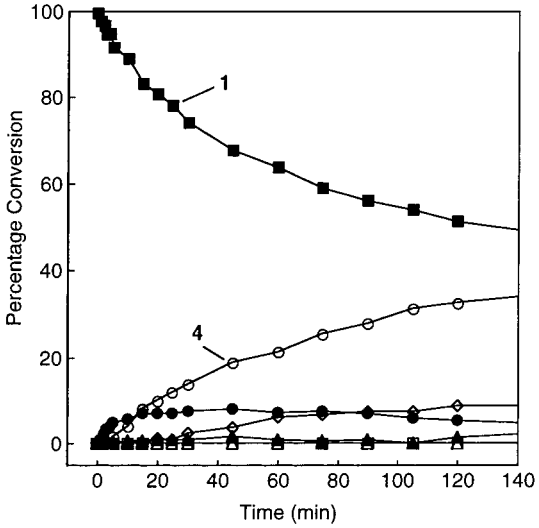

Figure 2. Effects of a MurD inhibitor on the murein pathway. Conditions and symbol references are the same as those in Figure 1 except that 10 nM L-805,170 was included in the reaction mixture. Note the accumulation of the substrate for MurD (**4**, (○)).

manner and induced an accumulation of **4** (Figure 2). However, L-805,170 did not inhibit MurE or MurF, showing that its effects on the pathway were due to inhibition of MurD. Neither did it possess antibacterial activity. Inhibitors of MurA (fosfomycin) and MurB (NADP$^+$) inhibited the pathway similarly (data not shown).

Although HPLC analysis permits a detailed accounting of the pathway intermediates, it is too cumbersome for high throughput screening (HTS). By running the pathway assay with radiolabeled **8** instead of **1**, only two radiolabeled species, **7** and **8**, need to be considered. Using AG1-X2, a rapid bind-and-elute separation of these two species was developed that is reproducible and amenable to automation.[9] In this assay format, the IC_{50}(L-805,170) = 9.7 ± 0.8 nM. Note that the IC_{50}(L-805,170) = 20 nM for *E. coli* MurD.[8] Once an inhibitor is detected by using the HTS pathway assay, the specific enzyme target(s) can be determined by using the individual enzyme assays.[10]

Apart from the obvious advantages of increased screening efficiency and sample conservation, this pathway assay may uncover inhibitory compounds that are unique to the gestalt of an intact pathway. Pathway enzymes have evolved conserved binding motifs to bind structurally related pathway metabolites. For example, the murein ligases MurC, MurD, MurE, and MurF are orthologous as well as paralogous and share a common kinetic mechanism involving ATP.[11] An inhibitor that recognizes homologous binding motifs will likely bind to more than one enzyme in the pathway. Thus, modest inhibition of several enzymes might reduce flux through the pathway more effectively than potent inhibition of a single enzyme. Furthermore, the frequency of target-mediated resistance to such a compound would be negligible, since mutations conferring resistance would need to occur in at least two different target genes during a single generation. Such a multimodal inhibitor can only be selected in a pathway context.

Operated in HTS mode, the murein pathway assay can efficiently interrogate an ensemble of validated antibacterial targets simultaneously using limited quantities of test compounds. Indeed, this approach can be applied to any therapeutically relevant metabolic pathway. Moreover, this assay will help pinpoint the enzymatic target(s) of antibacterial compounds discovered in whole cell assays.[12] Finally, screening an intact metabolic pathway may identify compounds that block multiple enzymes. Together with rapid analogue synthesis, the ability to screen enzymes "combinatorially" will accelerate the discovery of the next generation of antibiotics.

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(9) For high throughput bead-binding assay, pathway assay reactions (see ref 6) used [^{3}H]-D-alanyl-D-alanine or [^{14}C]-D-alanyl-D-alanine (400 nCi) as the radiolabeled tracer instead of [^{3}H]-**1**. Reaction mixtures were first preincubated with test compounds for 45 min at 25 °C before being initiated by adding unlabeled **1**. After 90 min, reactions were quenched with 50 μL of 500 mM KH_2PO_4, pH 3.5. Adjustment of reaction volume to 200 μL with water was followed by addition of AG1-X2 resin (100 μL; 1:1 (w/v) in 25 mM KH_2PO_4, pH 3.5), and incubation at 25 °C overnight. The resin was washed with water (200 μL, three times). Product was eluted with 50 μL of 1 M salicylic acid and radioactivity measured by liquid scintillation counting. Data were reported in percent inhibition compared to an uninhibited control. The add-and-mix parts of the assay were performed by the TECAN/Genesis system using a 96-well format. Separation of radiolabeled product from starting material using AG1-X2 beads was performed manually but can be semi-automated using the 96-well automated pipetting station Quadra 320 (TOMTEC).

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