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Identification of Compounds That Inhibit Late Steps of Peptidoglycan Synthesis in Bacteria

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A screening system is described that can detect and confirm inhibitors of the late steps of cell wall biosynthesis. The primary high through-put screen monitors induction of β-lactamase following exposure to samples, in an *Escherichia coli envA-* strain that carries the β-lactamase gene from *Citrobacter freundii* on a plasmid. Positive samples were detected from compound libraries, from natural products libraries, and from fractions of natural products crude preparations. These samples were then subjected to *in vitro* assays that could detect the incorporation of soluble cell wall precursor into Lipid I, Lipid II, and polymerized cell wall, using a TLC system that was very accurate and unambiguous in detecting known cell wall inhibitors. One partially purified sample containing a novel antibacterial agent derived from natural products was found to inhibit the formation of Lipid I (50% inhibition at $\leq$62.5 ng/ml), whereas another partially purified sample also derived from natural products inhibited transglycosylation into cell wall polymer (50% inhibition at $\leq$10 μg/ml). This screening system proved to be especially useful because it was sufficiently sensitive and robust to detect inhibitors among samples of crude preparations or varying states of purity.

Enzymes and structures involved in cell wall biosynthesis have proved to be excellent targets for antibacterial agents because the cell wall pathway is conserved among bacterial pathogens and is absent from mammalian cells. Inhibitors of several steps in cell wall biosynthesis have been identified, and more than forty cell wall active agents have been approved for clinical use[1]. The early steps in cell wall synthesis lead to the formation of the soluble precursor, UDP-muramyl-pentapeptide. The late steps involve the formation of the lipid-bound intermediates (Lipid I and Lipid II), transglycosylation into the polymeric chain, and transpeptidation, resulting in cross-links between chains[1~4]. Because of the membrane-bound nature of the reactions that occur late in the pathway, the late steps are more difficult to screen for biochemically, whereas biochemical screening of the early steps in wall biosynthesis have been described previously[1]. Our goal was to identify novel compounds that inhibit the late stage reactions leading to incorporation into the cell wall polymer. Our screening strategy allowed for the detection of inhibitors of the formation of Lipid I (carried out by the MraY protein), Lipid II (carried out by MurG), and transglycosylation (catalyzed by the large penicillin binding proteins).

In order to detect inhibitors of the late cell wall reactions, we refined the assays for detecting these biosynthetic steps by developing thin layer chromatography assays, called TLC System I and TLC System II. These assays could detect products from radioactive substrates and could measure the quantitative effect of inhibitors of these reactions. However, because the enzyme source for these assays was a particulate fraction, in common with other assays of these cell wall reactions that have been described[5~7], TLC Systems I and II were not amenable to high-throughput screening. Therefore a two-step strategy was employed. Initially samples were subjected to the β-

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lactamase induction screen that was capable of detecting activities that affected either early or late steps in cell wall biosynthesis[8]. The small minority of samples that tested positive in the β-lactamase induction screen were then subjected to the assays described here, which could detect late stage inhibitors and pinpoint the inhibited step.

Both chemical and natural products files were sources for active compounds. The partial characterization of two compound classes derived from natural products are described, one which inhibits the formation of Lipid I and the other which prevents transglycosylation.

Materials and Methods

β-Lactamase Induction Screen

Samples were tested for cell wall activity as described[8]. Briefly, growing cells of *Escherichia coli* strain D22 carrying plasmid pNU304 were exposed to samples, or the positive control compound, fosfomycin, in 96-well microtiter plates. After incubating for 1 hour at 37°C, 30 μl of cells were added to 90 μl of reaction buffer to assay β-lactamase. (Reaction buffer contained 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 246 mg $MgSO_4 \cdot 7H_2O$, 200 mg CTAB (hexadecyl trimethylammonium bromide), and 100 mg Na deoxycholate per liter, pH 7.0, to which a 1/50 volume of 25 mg/ml nitrocefin solution was added.) The OD at 490 nm was measured immediately and after 2 hours incubation at room temperature. The induction value was calculated by comparing the OD of each sample to the OD of induced cells as described[8].

MIC Testing

MIC testing was carried out by inoculating cells from a fresh LB agar plate into LB to a concentration of 5×10^5 cells per ml, in 100 μl cultures of a 96-well microtiter tray. The MIC was defined as the minimum concentration resulting in a cell density less than 0.01 OD increase compared to the zero-time reading (Biorad Model 3550 UV plate reader), which corresponded to no visible growth after incubating for 17 hours at 37°C.

TLC System I Assay for Late-stage Inhibitors of Cell Wall Biosynthesis

In order to detect inhibitors of late stage of cell wall biosynthesis, carried out by MraY, MurG, or transglycosylase, TLC System I was used. The membrane preparation catalyzing the reaction was prepared by harvesting *E. coli* K802 cells grown to 0.8 absorbance units (OD 600 nm). Cells were resuspended in 5 ml of cold lysis buffer (0.05 M Tris pH 8.0, 1 mM $MgCl_2$, and 2 mM β-ME) per gram of cells. Subsequent steps were carried out on ice or at 4°C. Cells were lysed using the Bead Beater (BioSpec 11079-00-101) with 4×2 minutes pulses with 2 minute rests in between. The cell lysate and beads were centrifuged at low speed followed by centrifugation at 10 kg for 20 minutes, and the supernatant was carefully removed and subjected to ultracentrifugation at 100 kg for 30 minutes. The pellet was resuspended in lysis buffer and adjusted to 7 mg protein/ml (determined by the Bradford protein assay).

The reaction volume contained a total of 30 μl and the assay was carried out at room temperature. First 10 μl of sample containing 30 μg/ml was added to 3 μl of 10× reaction buffer (consisting of 0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$, and 0.2 M KCl) and 7 μl of membrane preparation. After 10 minutes 5 μl of 2.5 mM UDPMurNAc-pentapeptide (cell wall precursor provided by Dr. PETER REYNOLDS, University of Cambridge) was added, and after 10 more minutes, 5 μl of 1.2 mM ^{14}C-UDP-GlcNAc (American RadioChemical, 300 mCi/mMol) were added. After 30 additional minutes incubation, samples were boiled for 1 minute to stop the reaction.

Samples of 2 μl were spotted on K6 Gel Silica TLC plates (Whatman), eluted with 50 : 30 isobutyric acid : 1 M NH_4OH. Kodak BioMax MS film with Kodak Transcreen LE was exposed to dried plates at −80°C overnight. Images of Lipid II (undecaprenyl-P-P-(GlcNAc-β1→4)-N-acetylmuramyl-pentapeptide), and the peptidoglycan product (found at the origin) were quantified by measuring the intensity of the appropriate spot after obtaining an image with a digital camera.

TLC System II Assay to Distinguish MraY and MurG Inhibitors

The membrane preparation was described in TLC system I. The substrates are the same as in TLC System I except that UDP-GlcNAc is excluded from the reaction mixture, and UDPMurNAc-^{14}C-DAP-pentapeptide (provided by Dr. TED PARK, Tufts University) was the radiolabeled substrate. The assay, at room temperature, was initiated when 10 μl of sample was mixed with 9 μl of membranes. After 10 minutes, 1 μl of UDPMurNAc-^{14}C-DAP-pentapeptide was added, and the reaction proceeded for 30 minutes. The reaction was arrested by boiling for 1 minute. The TLC protocol and the data computation for quantifying the formation of Lipid I (undecaprenyl-P-P-N-acetylmuramyl-pentapeptide covalently linked to the C55 isoprenoid (undecaprenyl-phosphate)), were as described above for TLC Assay System I.

LC-MS Analysis of Natural Products Extracts

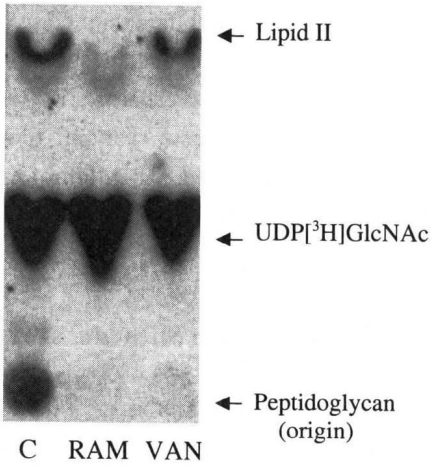
Extracts of active fermentation broths were treated and analyzed by suspending in a mixture of methanol/water (1 : 1 v/v), filtered, and subjected to HPLC as described[9], using conditions detailed previously[10].

Results

The TLC System I Can Detect Inhibitors of the Late Steps of Cell Wall Biosynthesis

The particulate preparation from *E. coli* cells, containing the proteins MraY, MurG, and transglycosylase, was capable of carrying out the late stages of cell wall biosynthesis, as shown in Figure 1. The control sample showed a clear separation of substrate and products using TLC System I. Labeled UDP-GlcNAc migrated from the origin, whereas Lipid II product, which eluted with greater facility because of the hydrophobicity of the isoprenoid moiety, migrated near the solvent front. The polymeric product of transglycosylation remained at the origin. Compounds that inhibited one of the late steps of cell wall biosynthesis in TLC System I exhibited one of two patterns. Ramoplanin, which is known to inhibit the MurG reaction[5], showed neither Lipid II product nor transglycosylation product. The transglycosylase inhibitor, vancomycin, completely inhibited the formation of transglycosylation (no spot at the origin), but had no effect on Lipid II formation.

Fig. 1. *In vitro* effects of cell wall inhibitors on late stage cell wall biosynthesis as detected by TLC system I.

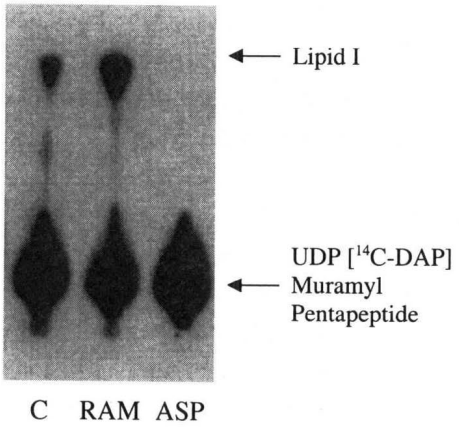


C: Control; RAM: Ramoplanin (30 μg/ml); VAN: Vancomycin (100 μg/ml).

TLC System II Specifically Assays Lipid I Formation

In the case of ramoplanin, the TLC System I, described above, did not distinguish if Lipid I or Lipid II formation was inhibited, because the migration distance of Lipid I and Lipid II were equivalent, and UDP-GlcNAc, which is a substrate for Lipid II formation, contained the radioactive label. In order to differentiate inhibitors of Lipid I and Lipid II formation, the TLC System II was developed. Samples were incubated with the particulate enzyme preparation as before, except that in TLC System II, labeled UDP-muramylpentapeptide was the only substrate in the reaction. Because UDP-GlcNAc was absent, the substrate could only be converted to Lipid I by the particulate enzyme preparation (Figure 2). In the presence of ramoplanin, there was no inhibition of Lipid I formation; in fact, there was a clear, reproducible enhancement in Lipid I formation. In marked contrast to ramoplanin, the cell wall inhibitor, aspartocin, prevented the formation of Lipid I, shown in Figure 2. Transglycosylase inhibitors such as vancomycin showed no activity in TLC System II, because they inhibit a step subsequent to the formation of Lipid I (data not shown). Thus the TLC System I can be utilized to detect late-stage cell wall inhibitors, and the TLC System II can be employed to distinguish inhibitors of Lipid I from inhibitors of Lipid II formation.

Fig. 2. *In vitro* effects of cell wall inhibitors on late stage cell wall biosynthesis as detected by TLC system II.



C: Control; RAM: Ramoplanin (30 μg/ml): ASP: Aspartocin (15 μg/ml).

The β-Lactamase Induction Screen Is an Effective System for Identifying Potential Inhibitors of the Late Steps of Cell Wall Biosynthesis

The TLC Systems I and II proved to be very useful in identifying late stage inhibitors. However, these assays were not ideal for running in high-throughput mode. Therefore we resorted to using the β-lactamase induction screen described in the accompanying paper[8], as the primary screen in our screening system. This cell-based screen was capable of detecting inhibitors of both the early and late stages of cell wall synthesis at low concentrations, with the virtue that it could be run in high throughput with facility. In order to make this cell-based screen sufficiently sensitive to larger molecules, the screening strain containing the *envA*- allele causing a hyperpermeable outer membrane[11,12], was used as described[8].

Samples from the Wyeth-Ayerst chemical file were subjected to the β-lactamase induction screen, as described in the Materials and Methods section. Samples from the Wyeth-Ayerst chemical file were resuspended in 50 mM HEPES buffer at pH 7.5, and 1/10 volume (10 μl) was added in each well to test for induction. The final concentration of test compounds was 12.5 μg/ml. When the Induction Value threshold was set at 0.2 (sufficient to detect 5% of the induction of fully induced cells[8]), then 1.03% of samples tested positive among more than 100,000 chemicals which were tested. The retest frequency was 54%. Among the positive compounds were moenomycin, avoparcin, and bacitracin, which were the control compounds in the file known to have cell wall activity.

The number of samples that were considered for TLC analysis was reduced 99%, showing that the β-lactamase induction screen had been very effective in discriminating against compounds with no cell wall inhibitory activity. Among hundreds of positive samples, 2.5% showed >50% inhibition of transglycosylation in the presence of 25 μg/ml of compound.

Screening Crude Extracts of the Millennium Pharmaceuticals Natural Products Library

The strategy for screening natural products for late stage inhibitors of cell wall synthesis was similar to the approach with the chemical file. The Millennium Pharmaceuticals natural products drug source is derived mostly from fungal fermentations. The crude extracts were first tested for cell wall activity using the β-lactamase induction screen[8]. Of the total of 1008 fungal isolates that were fermented, 170 tested and retested as positive cultures. The 65 cultures that tested positive in the β-lactamase induction screen following subsequent fermentations were subjected to fractionation as described in Materials and Methods, and each fraction of the sixty-five was tested for induction activity.

Extracts of isolates AA2495 and AA7798, two examples of the sixty-five, were both positive in the β-lactamase induction screen. Fractionation of the extract of culture AA2549 revealed that fractions 37~42 contained activity in the primary screen. When these fractions were run in TLC System I there was little or no effect on the synthesis of Lipid II, but there was a strong inhibitory effect on the polymerization of precursor into cell wall polymer, suggesting the presence of a transglycosylase activity (Figure 3). When an extract of isolate AA7798 was tested, it also had strong inducing activity. Fractionation revealed the strongest inducing activity in fractions 40~43. When these fractions were tested by TLC System I, there was inhibition of the formation of Lipid II as well as incorporation of label into cell wall polymer, suggesting an inhibitor preventing the formation of Lipid I or Lipid II (Figure 4).

Characterization of Natural Products from the Wyeth-Ayerst Natural Products Collection

When a collection of partially purified extracts of the Wyeth-Ayerst natural products collection were tested for activity in the β-lactamase induction screen, 5.3% tested positive in the β-lactamase induction screen when cells were exposed to 12.5 μg/ml of samples. Those extracts that tested positive were examined further for inhibition of late steps in cell wall synthesis. Figure 5 shows that one compound class called AC98 inhibited transglycosylation without any apparent effect on Lipid II formation. A concentration profile of AC98 revealed that the IC_{50} for AC98 was 10 μg/ml. Because the extract was partially purified, 10 μg/ml represents the minimum potency of the active component(s) in the mixture. The AC98 preparation showed antibacterial activity against Gram-positive microorganisms in the range of its enzymatic inhibitory activity against the Gram-positive bacteria *Staphylococcus aureus* (data not shown), suggesting that the cell wall activity was the source of its antibacterial activity.

When AA896 was similarly tested in TLC System I, it not only prevented the incorporation label into cell wall polymer, but also inhibited the formation of Lipid II, with an IC_{50} of 62.5 ng/ml (Figure 6). To determine whether Lipid I or Lipid II formation was affected, AA896 was run in TLC System II. AA896 showed potent *in vitro* inhibitory

Fig. 3. Identification of an inhibitor of transglycosylation in HPLC fractionated extract AA2549 using TLC system I.

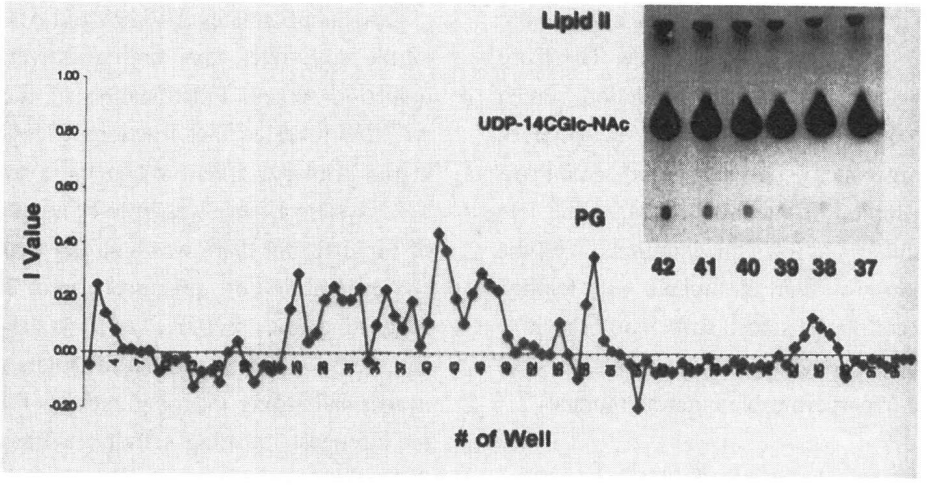
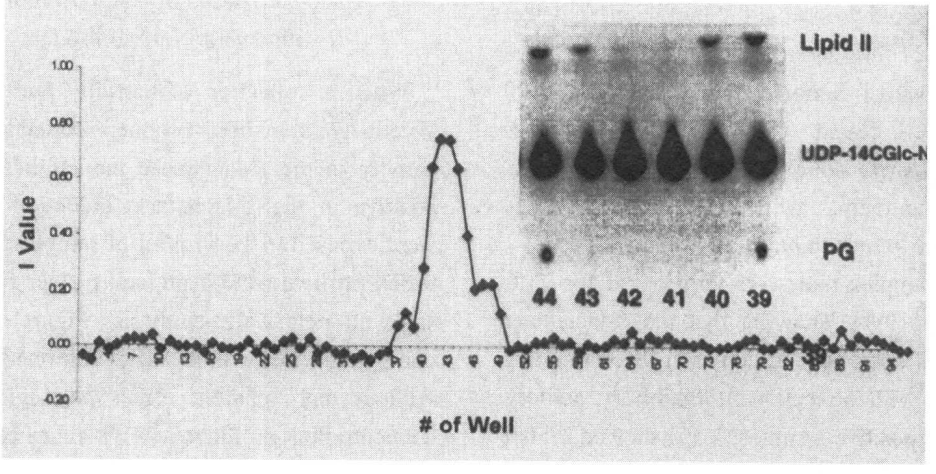


Fig. 4. Identification of an activity inhibiting both Lipid II and peptidoglycan formation in HPLC fractionated extract AA7798 using TLC system I.



activity in preventing Lipid I formation, with an IC_{50} of 2.5 μg/ml (Figure 7). Therefore AA896 affected cell wall synthesis by inhibiting the MraY reaction. The IC_{50} for Lipid II formation was approximately the concentration necessary for growth inhibition of *S. aureus* (data not shown), suggesting that the inhibition of Lipid I formation was the mechanism of growth inhibition.

Discussion

We describe a system to identify novel products that inhibit the late stages in cell wall biosynthesis, the formation of Lipid I and II precursors, and transglycosylation. These are proven targets of drugs. Although nisin[13] and mureidomycins[14] have been identified previously as inhibitors of Lipid I formation, ramoplanin[5] and tunicamycin[15] of Lipid II, and vancomycin[2] and moenomycin[2], as well as mersacidin[16],

and azureomycin B[17] of transglycosylation, we think that these steps in the pathway have not been exhausted in terms of chemically diverse compounds that could be effective antibacterials. It is possible that these late steps of cell wall biosynthesis, in particular, have been less thoroughly examined compared to the early steps of cell wall biosynthesis, because they are more difficult to assay effectively[1]. On theoretical grounds, the formation of Lipid I and Lipid II may be particularly vulnerable to inhibition, considering the small number of sites that carry out these reactions, which are approximately 700 Lipid I sites and 2000 Lipid II sites per cell[5,18,19]. Thus very little of an effective inhibitor may be required to kill a cell.

It is very difficult to screen directly in high throughput mode for such inhibitors. The TLC System I proved to be an excellent method to identify those inhibitors that prevented the formation of Lipid I, Lipid II, or cell wall polymer. There are other systems that have been described[5~7], but these systems, too, have the limitation that they are used most effectively as assays, not as screens. TLC System I presents a clear picture that can be effectively quantitated to obtain IC_{50} values from the area of the image.

Our strategy was to utilize a cell-based, pathway specific screen as the primary means of identifying candidate compounds[8]. The β-lactamase induction screen was effective because it satisfied the following criteria: (1) it was sensitive in detecting a wide variety of compounds that affect all cell wall biosynthetic steps, (2) it was effective in discriminating against most non-cell wall activities, and (3) it had a low background of false positive activities. Thus the concentrated effort could be devoted to the small

Fig. 5. Isolated natural product AC98 inhibits transglycosylation activity.

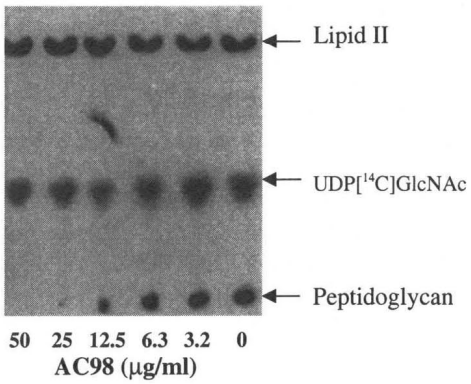


Fig. 7. Isolated natural product AA896 inhibits the formation of Lipid I using TLC system II.

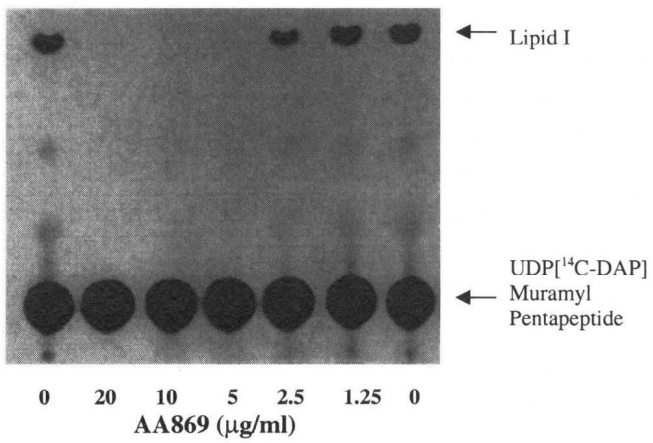
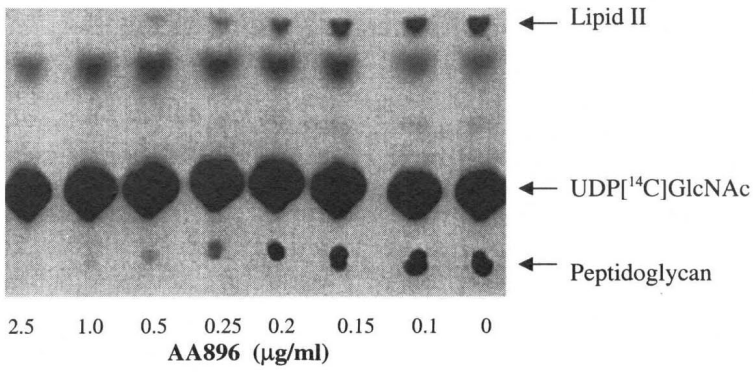


Fig. 6. Isolated natural product AA896 inhibits both the formation of Lipid II and peptidoglycan formation as monitored by TLC System I.



fraction of samples that were positive for induction. It was possible that an interesting pharmacophore could have been identified with this screening system from the chemical file that could have been used as a starting point for improvement with the aid of medicinal chemistry. However, the more promising lead candidates came from natural product samples.

Those compounds that prevented the synthesis of Lipid II in TLC System I could be run in TLC System II to distinguish those compounds that inhibited Lipid I and Lipid II formation. Ramoplanin is an interesting case in point. Figure 1 shows that ramoplanin prevented the formation of both cell wall polymer and Lipid II formation. Figure 2 shows that ramoplanin did not interfere with TLC I formation, and therefore must have inhibited the formation of Lipid II. In fact, Figure 2 shows that ramoplanin actually increased the formation of Lipid I, as has been noted previously[5]. Taking into account the observation that the Lipid I reaction is reversible[5], the most straightforward explanation for the apparent enhancement of Lipid I formation in the presence of ramoplanin is that the compound binds directly with Lipid I, stabilizing its formation, and arresting its conversion to Lipid II. Thus ramoplanin may inhibit cell wall biosynthesis by binding to a cell wall precursor structure, analogous to the action of vancomycin on Lipid II.

The TLC Systems I and II were less effective in detecting inhibitors of isoprenoid recycling, such as bacitracin, which shows a partial inhibition in TLC system I. Apparently the isoprenoid carried out some cycling in the *in vitro* system, sufficient to detect a partial effect on Lipid II formation (data not shown).

The discovery of activities from extracts of natural products suggests that the late stage reactions of cell wall biosynthesis remain viable targets, despite the fact that the cell wall pathway was the target for the first cell-based screens more than thirty years ago[2]. The use of a more sensitive pathway specific screen[8] may have aided in the detection of activities from the natural products crude and partially purified extracts presented here. The fact that AA896 and AC98 have novel structures (data not shown), provides additional support that novel inhibitors of the late stages of cell wall biosynthesis are still to be discovered. The development of the TLC Systems I and II assures that candidate samples can be identified as inhibitors of a specific late step of cell wall biosynthesis with accuracy and dependability.

Acknowledgments

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