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Benzylidene Rhodanines as Novel Inhibitors of UDP-*N*-Acetylmuramate/L-Alanine Ligase

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Abstract—Benzylidene rhodanines are novel inhibitors of UDP *N*-acetylmuramate/L-alanine ligase. They showed selective whole-cell activity against the Gram-positive MRSA but not against the Gram-negative *Escherichia coli*. Their cytotoxic effect on mammalian CHO cells was also evaluated.

In the last decade, the emergence of both vancomycin and methicillin resistant bacteria[1] has led to the search for novel class of antibiotics as well as new targets susceptible to inhibition. One of the most effective antibacterial strategies is by disrupting the integrity of bacterial cell wall formation. Peptidoglycan is an essential component of the cell walls of both Gram-positive and Gram-negative bacteria. The peptidoglycan layer is responsible for the bacterial cell wall strength. The biosynthesis of peptidoglycan includes four steps in which successive amino residues are ligated to uridine diphospho-*N*-acetylmuramic acid (UDP-MurNAc). Each step is catalyzed by an enzyme which belongs to the Mur synthetases. Of these enzymes, the UDP-MurNAc/L-Ala ligase (MurC),[2] encoded by *murC* gene, catalyzes the formation of the first peptide bond between UDP-MurNAc and L-alanine with concomitant hydrolysis of ATP into ADP and inorganic phosphate (Fig. 1).

The reaction mechanism is believed to involve an acylphosphate intermediate, which undergoes the nucleophilic attack by alanine to yield a tetrahedral intermediate.[3] This intermediate breaks down eventually into UDP-MurNAc-L-Ala and inorganic phosphate. These ligases are highly specific and present only in eubacteria, thus making them extremely attractive as new targets for development of therapeutic agents against bacterial infection.

In a systematic search for new antibacterial agents from our inhouse chemical libraries, we discovered benzylidene rhodanine[4] **1** ($IC_{50} = 27$ μM) (Table 1) as a promising inhibitor of MurC through high throughput screening. Based on the structure of benzylidene rhodanine **1**, about 40 analogues were eventually synthesized and tested in the MurC assay.

Of all the analogues tested, only 5 compounds (**2**–**6**) were found to inhibit MurC (IC_{50} 12–24 μM, Table 1). Compounds **2**[5] (removal of a CH_2 group) and **3** (replacement of S with O) exhibited comparable activity as benzylidene rhodanine **1**. Compounds **5** and **6**, which have different benzylidene substituents at the C-5, were found to have approximately 3- and 1.5-fold respectively, improvement in activity.

No activity was found in compounds that were substituted at the N-3 position (methyl, ethyl, allyl, aryl, methylacetyl) of benzylidene rhodanines **1**, **3** and **6**. In fact, no activity was observed in all *N*-substituted compounds, thus suggesting the importance of a free NH group which might be required for hydrogen bonding at the MurC active site.

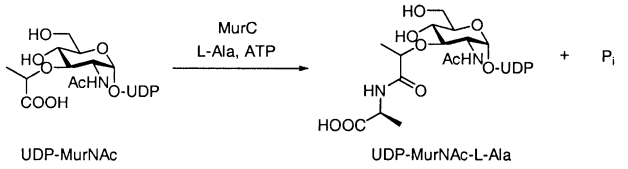

Figure 1. Enzymatic synthesis of UDP-MurNAc-L-Ala.

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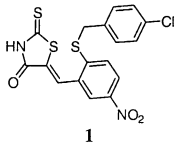
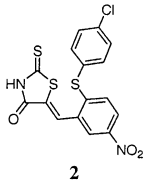
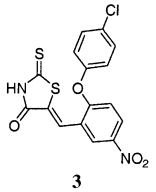
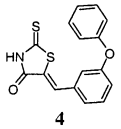
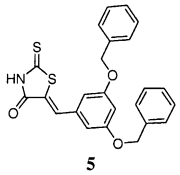
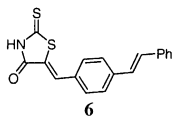
In contrast, there appeared to be no stringent requirement for the aryl substituent at the C-5 methylene of benzylidene rhodanine **1**. However, activities were observed in analogues with phenoxy-, benzyloxy-, phenylsulfanyl- or benzylsulfanyl-benzylidene and not in analogues with alkyl- or nitro-benzylidene, furanylmethylene, halo-, alkyl-, or aryl- furanylmethylene, alkoxy-naphthalenylmethylene, indanylmethylene, or biphenylethylidene.

The whole cell inhibitory activity of benzylidene rhodanines **1**–**6** was also evaluated. All of them were not active against the Gram-negative *Esherichia coli*, whereas benzylidene rhodanines **1**, **2** and **4** showed modest inhibition against Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA). Of these three compounds, benzylidene rhodanine **1** was toxic to Chinese hamster ovary (CHO) cells at 29 μM.

A similar finding was reported by Foye and Tovivich.[6] In their paper, only rhodanine and 5-(2-nitrobenzylidene) rhodanine showed extremely weak activity (mM) against *E. coli*, while some 5-substituted rhodanines were active against *S. aureus*. Furthermore, they mentioned that 3- or 5-substituted rhodanines were important for antimicrobials activities, and the presence of nitro group was essential for high activity (MIC ca. 10 μM for 5-(2-nitrobenzylidene) rhodanine). In our study, we have shown that 3-substituted rhodanine was detrimental to the activity and many 5-(nitrobenzylidene) rhodanines did not inhibit MurC. Furthermore, compound **4**, which was as potent as the nitro-containing rhodanines **1**–**3**, was also active in the whole cell assay.

During the process of our work, Grant-Young and co-workers reported 2,3,5-trisubstituted-4-thiazolidinone as novel MurB inhibitors (IC_{50} 8–28 μM).[7] These findings further supported the fact that thiazoldinones were long known for their antibacterial activities.[8] Reck and co-workers reported an in vitro phosphinate MurC inhibitor ($IC_{50} = 0.05$ μM) recently.[9] Biochemical char-

Table 1. MurC inhibition, whole-cell inhibition and cytotoxicity effect of benzylidene rhodanines **1**–**6**

Compd	MurC inhibition IC_{50} (μM)	MRSA inhibition MIC (μM)	*E. coli* inhibition MIC (μM)	CHO inhibition IC_{50} (μM)
 **1**	27	31	>65	29
 **2**	24	70	>87	>73
 **3**	24	>76	>76	Not determined
 **4**	22	79	>87	>96
 **5**	12	>69	>69	Not determined
 **6**	18	>93	>93	Not determined

acterization of this inhibitor showed that it exhibits mixed-type inhibition with respect to all three enzyme substrates.[10] Although our benzylidene rhodanines **1**–**6** were modest inhibitors of MurC, 3 of them exhibited selective inhibition in whole cell Gram-positive MRSA. Furthermore, the ease of synthesis and the availability of building blocks provide an attractive route toward the discovery of more potent MurC inhibitors.

Materials and Methods

Microtitre plates were purchased from Greiner Labortechnik, Germany. Mueller-Hinton (MH) broth was from Oxoid. Fetal bovine serum was from Hyclone Laboratories Inc., Logan, UT. Malachite Green was from Carlo Erba Reagenti, Italy. All other chemicals were purchased from Sigma Aldrich Chemical, St. Louis, MO, USA. CHO cells were purchase from American Cell Type Culture Collection (Rockville, MD, USA). *S. aureus* COL, *E. coli* 1852E, recombinant MurC enzyme and UDP-MurNAc were provided by GlaxoSmithKline.

MurC enzyme assay

Compounds were incubated with MurC in a reaction mixture containing 6 μg/mL MurC, 0.1 mM UDP-MurNAc, 1 mM ATP, 1 mM L-alanine, 100 mM Tris pH 8.8, 20 mM $MgCl_2$, 1 mM DTT. After 30 min incubation at room temperature, Pi reagent (0.325g/L Malachite Green, 4.64 g/L polyvinyl alcohol and 11.44 g/L ammonium molybdate) was added. Plates were read at 600 nm after 5 min incubation with Pi reagent.

Bacterial whole cell assays

Bacteria from overnight culture were diluted 100 times with fresh MH broth and allowed to reach the log phase of growth at 100 rpm, 37 °C. The bacterial cultures were then diluted to obtain a final inoculum size of 2.5×10^5 cfu/mL in MH broth. 90 μL of the adjusted inoculum was then dispensed into 96-well U-bottom polystyrene plates containing 10 μL of test compounds in 12.5% DMSO. Plates were then incubated overnight at 37 °C. After 18–20 h incubation, 50 μL of 0.032% resazurin in MH broth were added to each well. Plates were then incubated at 37 °C for 30 min. Fluorescence measurements were taken by exciting at 530 nm and determining the fluorescence density at 590 nm.

Mammalian cell cytotoxicity

CHO cells were seeded at a density of 12,000 cells/well in 100 μL of Ham's F12 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Plates were incubated overnight at 37 °C in the presence of 5% carbon dioxide. On day 2, the growth medium was replaced with 90 μL of Ham's F12 medium supplemented with 2% FBS, 2 mM L-glutamine, 50U/mL penicillin and 50 μg/mL streptomycin and 10 μL of test compounds in 12.5% DMSO was then added to the wells. After 24 h incubation, 25 μL of 0.05% resazurin was added to each well and the plates incubated for another 1 h before measuring fluorescence density at 590 nm.

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