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Synthesis of azide derivative and discovery of glyoxalase pathway inhibitor against pathogenic bacteria¹

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Abstract

A Glyoxalase inhibitor was synthesized and tested against *Staphylococcus aureus* for first time and showed MIC₉₀ of 20 µg/ml. Henceforth, we synthesized unnatural azide derivative of the same inhibitor to improve the biological activity. In that order, an azide carboxylate was synthesized from dimethyl tartrate by tosylation and azide substitution. The synthesized, azide compound was coupled with glutathione derivative in high yield and tested against *S. aureus* and showed improved MIC₉₀ of 5 µg/ml. In general, it can be also easily converted to unnatural - amino acid in good yield. The shown methodology will be extended to study induced suicide in *Burkholderia mallei*, *Francisella tularensis* and *Mycobacterium tuberculosis* in future.

Keywords

Glyoxalase pathway; Metabolism; *Staphylococcus aureus*; Glutathione derivative; Inhibitor

Approximately 10 million people die every year due to bacterial infections and no new classes of antibacterial drugs have been produced since 1980's¹. The numbers of drug resistant pathogen strains are also increasing;^{2,3} therefore new active antibacterial drugs⁴ are necessary.

Recently, glyoxal pathway has been shown more interest and tested in *E. coli*,⁵ *P. falciparum*,⁶ and *A. thaliana*⁷ and has led to identification of growth inhibitors.

In glycolytic metabolism, methylglyoxal (MG) is produced as a byproduct, which is a highly toxic -oxoaldehyde and an unavoidable compound in microorganisms. MG modifies arginine, lysine and cysteine residues in proteins, forming glycation end products and impairing the biochemical functionality of those macromolecules. MG also reacts with guanyl nucleotide in nucleic acids forming adducts that can lead to DNA modification such as DNA adducts, mutations, chromosomal aberrations, DNA repair, sister chromatid exchanges and DNA single strand break. These processes ultimately lead to cell death. In the glyoxalase pathway, methylglyoxal is detoxified by reacting with glutathione to form hemithioacetal (HTA) (Fig. 1)⁸. HTA is then converted to *S*-D-lactoglutathione (SDLGSH) by GlxI. SDLGSH reacts in the presence of GlxII to form D-lactate and regenerates glutathione. The glutathione is then reused or recycled. The explained glyoxalase pathway is

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the major pathway for detoxification of MG.^{9–11} Even though GlxI and GlxII are well known in gram negative bacteria, they are not well explored in gram positive bacteria. Recently, GlxI enzyme is reported in gram positive bacteria - *Clostridium acetobutylicum*.¹² Interestingly, the evidence for the functional protein has been well determined for the same microorganism. Most importantly, the same glyoxal pathway is not found in human cells. Therefore, it is considered to be a good drug target for the development of antimicrobial, antimalarial and herbicidal agents, a hypothesis not explored by many researchers. Here, we report the synthesis and inhibition of glyoxalase pathway inhibitor against *Staphylococcus aureus*.

N-phenylmethoxy carbonylglutathione^{13,7,14} a known *A. thaliana* GlxII inhibitor, **2** was synthesized from glutathione, **1** by reacting with benzyl chloroformate (Scheme 1). The synthesized compound, **2** was purified and characterized by ¹H-NMR and MS and tested against *Staphylococcus aureus*, and *Burkholderia thailandensis* for the first time. The synthesized compound, **2** showed MIC₉₀ 20 µg/ml on testing against *Staphylococcus aureus* and 12.5 µg/ml against *Burkholderia thailandensis* respectively (Table 1).¹⁵ This promising preliminary result displayed strong support for improving the glyoxalase pathway inhibitor using induced suicide methodology for *Staphylococcus aureus* and *Burkholderia thailandensis*.

Earlier it was observed that the presence of α -amino acid at the terminal end enhances the biological activity of many compounds.¹⁶ Therefore, we decided to link azide carboxylate group, which is similar to the α -amino acid group, to the free terminal of the known inhibitor, **2** to increase the biological activity. The azide compound, **5**¹⁷ was synthesized from the available dimethyl tartrate (Scheme 2). Briefly, commercially available dimethyl *L*-tartrate, **3** was activated by monotosylating the single hydroxyl group with one equivalent of tosyl chloride in the presence of pyridine, triethylamine and catalytic amount of DMAP (dimethyl amino pyridine) in dichloromethane. Concurrently, the azide group was introduced by nucleophilic substitution reaction using sodium azide. The azide ion displaced the tosyl group by S_N2 reaction in DMF solvent. The synthesized unnatural azide derivative, **5** was successfully coupled with the known inhibitor, **2** to produce unnatural glutathione derivative, **6**. Briefly, HATU ([Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) was used to activate the terminal primary carboxylic acid group in **2** in presence of triethylamine. Fortunately, the secondary carboxylic acid group in glutathione derivative, **2** was not affected because of more steric hindrance. The newly synthesized **6**, was characterized and confirmed by NMR and MS¹⁸ and tested against *Staphylococcus aureus* and showed MIC₉₀ of 5 µg/ml (Table 1). The same compound was also tested against *M. smegmatis* and observed MIC₉₀ of 6 µg/ml. Since the compound **6** showed better activity against *S. aureus* we were interested in evaluating the glyoxalase enzyme in *S. aureus*. Interestingly, through BLAST analysis we found that putative *S. aureus* glyoxalase had 47 % identity with *E. coli* glyoxalase and encoded a polypeptide of 229 amino acids with a molecular weight of 28.9 kDa. Designing and synthesis of novel compounds are in process to improve the biological activity against *S. aureus*.

The toxicity was tested by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in PBMC (peripheral blood mononuclear cell) cells. For determination of cytotoxicity of the inhibitors, MDM (monocyte derived macrophages) were cultured and treated with compounds at 0.05 mM for 8 hours at 37°C in 5% CO₂. Following loading of each inhibitors, cells were washed with serum-free culture medium to remove excess compounds. The cytotoxicity was assessed over the subsequent 24 hours using the alamar Blue TM assay (AbD Serotec, Raleigh, NC).¹⁹ Interestingly, both the inhibitors, **2** and **6** are

non-toxic and showed strong potential to be developed as drug candidate for future lead optimization and trials.

Hereby, we successfully synthesized unnatural azide carboxylate, **6** from commercially available dimethyl tartrate, **3**. In addition **6** can be also easily converted to unnatural α -amino acid in good yield. We coupled **5** with the known glyoxalase pathway inhibitor, **2** to generate a novel compound, **6**. The resulted azide glutathione derivative, **6** was tested against *Staphylococcus aureus* for the first time and observed moderate biological activity of MIC₉₀ of 5 μ g/ml as a glyoxalase pathway inhibitor. Presently, lead optimization is undergoing in our lab and in future it will be extended to study induced suicide in *Burkholderia mallei*, *Francisella tularensis* and *Mycobacterium tuberculosis*.

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15. 96-well U-bottom assay plates were seeded with 1×10^6 CFU in a volume of 0.1 mL/well for *Staphylococcus aureus* assays. Testing compound was serially diluted in DMSO as 1:2 dilutions in a separate 96-well plate and then transferred to the assay plate. The final concentration of DMSO in the assay plates did not exceed 2% (*S. aureus*). Plates were incubated at 37°C for 24 hours (*S. aureus*). O.D.600 readings were taken once at the end of the 24-hour incubation for *S. aureus*. Sample wells exhibiting 20% or less of the average O.D.600 readings of the untreated control wells were considered to contain a drug concentration resulting in inhibition. Alamar Blue dye solution was added to the wells at the end of the incubation period to a final concentration of 10% (v/v) and assessed after 1 hour (*S. aureus*) to determine the point of activity. Each MIC assay included untreated controls, media blanks, and control compounds.

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18. Triethylamine (6 μ L, 0.044 mmol, 1.0 equiv.) and HATU (16 mg, 0.044 mmol, 1.0 equiv.) were added to a solution of the tripeptide (20 mg, 0.044 mmol, 1.0 equiv.) in DMF (0.5 mL) at 0 °C under argon. A solution of the azide (9 mg, 0.044 mmol, 1.0 equiv.) and triethylamine (6 μ L, 0.044 mmol, 1.0 equiv.) in DMF (0.5 mL) was then added dropwise. The mixture was stirred and gradually warmed to rt overnight and concentrated. The residue was diluted with EtOAc (10 mL) and washed with 1M HCl. The aqueous layer was extracted further with EtOAc (3 $\times$ 10 mL). The organic extracts were combined, dried over Na₂SO₄ and concentrated. The residue was purified by flash chromatography eluting with 9:1 CH₂Cl₂/CH₃OH to give compound **6** (9 mg, 35 %). R_f 0.40 (9:1 CH₂Cl₂/MeOH). ¹H NMR (500 MHz, CD₃OD) 1.90–2.40 (m, 4H), 2.81 (s, 3H), 2.86 (s, 3H), 3.0–3.60 (m, 3H), 4.0–4.60 (m, 3H), 4.80–5.40 (m, 4H), 7.10–7.60 (m, 5H). ¹³C NMR (125 MHz, CD₃OD) 25.2, 28.1, 33.1, 41.9, 53.2, 53.3, 57.5, 58.3, 63.9, 65.2, 83.1, 128.0, 128.3, 128.9, 129.4, 129.5, 135.9, 156.7, 171.8, 172.8, 175.4. MS (m/z) calcd for C₂₄H₃₀N₆O₁₂S (M + H)⁺: 626; obsd: 626.
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Fig. 1.
The glyoxalase pathway

Scheme 1.
Synthesis of **2**

Scheme 2.
Synthesis of **6**, an unnatural glutathione derivative

Table 1

MIC values against microorganisms

Compound	Microorganism	MIC ₉₀ (µg/ml)
2	Burkholderia thailandensis	12.5
2	Staphylococcus aureus	20.0
6	Mycobacterium smegmatis	6.0
6	Staphylococcus aureus	5.0