

# Non-Natural D-Amino Acids to Control Bacterial Virulence

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## Introduction

The progressive slowing of antibiotic development and the increase of bacterial drug resistances poses a major threat to human health [1]. Bacteria aggregate to form potent virulent communities bound by an extracellular polymeric substance composed of sugars, proteins, and other secreted biomolecules. The resultant structure, a biofilm, is responsible for an estimated 80% of known bacterial infections. This formed barrier is resistant to changes in temperature, salinity, and antimicrobial agents, protecting proliferating cells on the interior of the structure. Thus, dismantling biofilms can serve as a means of impeding pathogenicity [2].

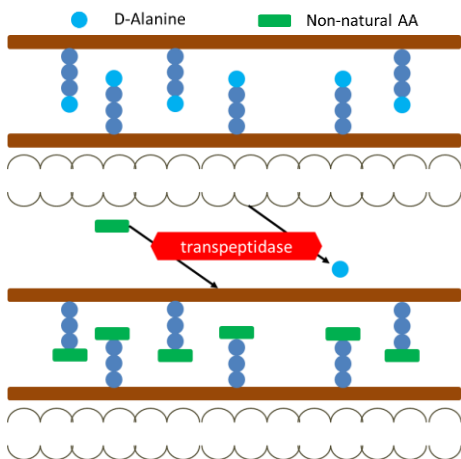


Fig. 1. The incorporation of non-natural amino acids into the peptidoglycan layer of the gram-positive bacterial cell wall.

In 2010, Kolodkin-Gal and colleagues reported that D-amino acids are potent biofilm disruptors in gram-positive bacteria [3]. It was suggested that the terminal D-alanine residue of the gram-positive peptidoglycan layer can be replaced by another native or non-native amino acid *via* the promiscuous enzyme transpeptidase (Figure 1). The resultant substitution interferes with protein synthesis, leading to the eventual demise of the cell. This and other subsequent studies have divulged several amino acids capable of effective biofilm disassembly at low micromolar concentrations, with D-tyrosine as one of the most potent [4]. However, no clear correlation between amino acid structure and activity has emerged. Further,

only the enantiomers of the twenty canonical amino acids have been screened, providing optimal space for the development of synthetically modified side chain residues to enhance potency.

In this study, we report a preliminary investigation into synthetic D-amino acids for biofilm disruption. Modified from a parent glutamic acid structure, three novel amino acids were prepared and screened in cultures of the opportunistic pathogen *S. epidermidis* to probe their antimicrobial capabilities.

## Results and Discussion

To expediently develop a small set of novel D-amino acids for screening, glutamic acid was used as a handle for coupling a variety of primary and/or secondary amines to afford a *de novo* amide bond. Inspiration for the selection of amine coupling partners was taken from previously reported biofilm disruptors, like D-tyrosine and D-leucine that contain aromatic or larger aliphatic side chains (Figure 2) [4]. The amines were added to a cooled solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), triethylamine, and Boc-D-glutamic acid methyl ester to yield the corresponding protected amino acid. In the presence of concentrated hydrochloric acid, the Boc and methyl ester moieties were cleaved to produce the free amino acid.

Solutions of the purified amino acids (0.4  $\mu$ M to 400  $\mu$ M) were prepared by serial dilution and combined with *S. epidermidis* biofilms, grown for 24 h, in a simple crystal violet well-plate assay. After another 24 h incubation, the plates were read at 580 nm to determine the minimum inhibitory concentration of each synthetic amino acid. The combined data from 8 replicate trials reveals only modest variation in the biofilm surface (Figure 3). Both KM01 and the positive control D-tyrosine

displayed a small but global reduction in absorbance, while KM02 and EM01 showed no significant decrease in biofilm density relative to the control. The discrepancy in the activity from D-tyrosine and KM01 to KM02 implies that the carboxylate moiety in KM02 reduces biofilm disassembly, perhaps indicating the benefit of an electron-rich arene.

However, a weak correlation was displayed between amino acid concentration and antibiofilm activity. In addition, the magnitude of biofilm reduction in all cases is minimal compared to the degree of biofilm disassembly described in previous reports. Amino acids, such as D-tyrosine, were shown to eradicate biofilms at concentrations as low as 10  $\mu\text{M}$ , which was simply not represented in our control experiments [4,5]. A study published concurrently by Sarkar and colleagues cites similar difficulties obtaining comparable potencies as those initially reported for D-amino acid mediated biofilm disassembly. After screening 96 natural and non-natural D-amino acids in three species of gram-positive pathogens (*S. epidermidis*, *S. aureus*, and *B. subtilis*), no significant biofilm reduction was observed [5]. These results call into question the effectiveness of employing D-amino acids as antimicrobials.

Despite the dissention in the field regarding the potential of D-amino acids to serve as biofilm disruptors, the pathway for the incorporation of non-natural amino acids into the peptidoglycan layer of gram-positive bacteria has been verified. The well-known epitope 2,4-dinitrophenylalanine (DNP) was introduced into the internal oligopeptides of *B. subtilis*, promoting assembly of endogenous antibodies at the cell surface [6]. The flexibility of the transpeptidase enzyme mediating this process

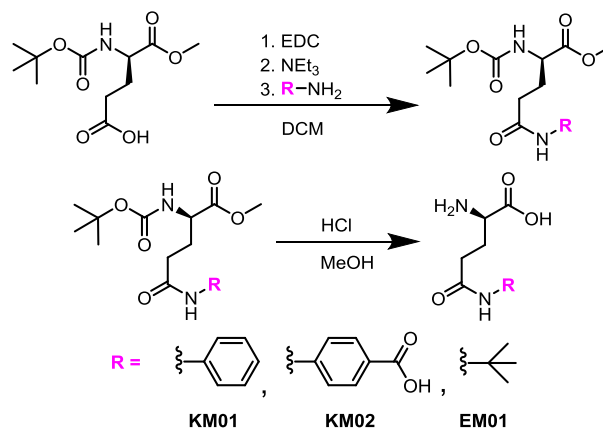


Fig. 2. Scheme for the synthesis of D-glutamic acid analogs KM01, KM02, and EM01.

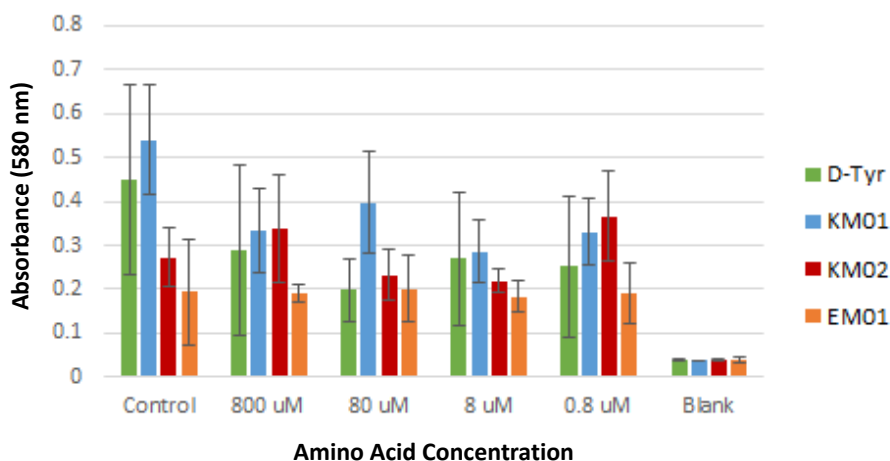


Fig. 3. Decrease in *S. epidermidis* biofilm density (absorbance) after incubated for 24 h with D-tyrosine and three D-glutamic acid derivatives KM01, KM02, and EM01.

was further employed to modify extracellular peptides at their C-termini, largely altering the topography of the bacterial cell wall [7].

In this initial investigation of synthetic D-amino acids as biofilm disruptors, three novel glutamic acid derivatives were designed and screened in *S. epidermidis*. Although the analogs only displayed slight perturbations in biofilm densities, the feasibility of inserting synthetically modified amino acids into the gram-positive cell wall has been verified in the literature. By integrating previous research in peptide science and chemical biology in bacteria, the peptidoglycan layer remains an open canvas for antimicrobial decoration.

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