Substrate Analogs for Characterizing the Substrate Tolerance of *S. pneumoniae* Sortase A

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Abstract

Bacterial sortases have been widely studied for their utility in protein modification, however, the variable substrate specificity and activity between homologs of these enzymes is not yet fully characterized. To further understand sorting signal recognition, we are working towards a substrate bound structure of sortase A from *Streptococcus pneumoniae* ($SrtA_{pneu}$). This enzyme displays a wide tolerance for alternate amino acids within the canonical LPXTG sorting motif. Our strategy involves a non-cleavable substrate analog that can be docked into the active site, allowing for elucidation of a structure displaying the key contacts that allow the enzyme to recognize alternate sorting signals. To this end, ketomethylene-linked "dipeptide" isosteres were synthesized and incorporated into peptides via solid phase synthesis to produce non-cleavable sorting signals accepted by $SrtA_{pneu}$. These substrate analogs were found to inhibit $SrtA_{pneu}$ activity in a model transpeptidation reaction.

Background

Sortase A from *S. aureus* recognizes an LPXTG (X = any amino acid) motif, which it binds and cleaves to form a thioester-linked enzyme substrate complex. This intermediate is then attacked by a nitrogen nucleophile (*in vivo* this is lipid II) which transfers the peptide to the nucleophile, forming a new adduct and restoring the active site cysteine. This function has gained recognition for its usefulness in protein modification, as well as its necessity for bacterial cell virulence. Recently, our lab has determined that $SrtA_{pneu}$ is particularly promiscuous with regards to its substrate preferences. A peptide library with the general sequence LPATZG (Z = randomized residue) was synthesized and screened against $SrtA_{pneu}$. Reactivity was observed for numerous substrates with variable residues in the fifth position. In addition to providing new substrates for protein modification chemistry, this data also raises questions regarding the ability for the enzyme to accept non-canonical substrates. Specifically, we are interested in the structural basis for the broad substrate tolerance of $SrtA_{pneu}$.

Sortase A homolog substrate promiscuity data

Abz-**LPATZ**G-K(Dnp) —sortase A, H_2N-OH→ Abz-**LPAT**-NHOH + **Z**G-K(Dnp)

sortase homolog	Z = V	Y	S	W	L	Nle	G	A	N	F	Q	C
S. aureus	3	1	1	0	0	0	86	2	0	1	2	5
S. suis	2	2	67	0	0	2	71	74	58	3	3	73
S. oralis	2	1	54	0	0	1	74	46	3	2	6	48
S. pneumoniae	39	14	84	11	41	14	81	92	48	29	11	76
L. monocytogenes	4	43	41	6	1	2	86	15	22	30	1	42
E. faecalis	1	0	7	0	0	1	7	5	6	3	1	10
L. lactis	5	1	44	0	1	1	83	66	28	2	1	32
B. anthracis	1	2	8	0	0	1	42	22	17	1	0	17
L. plantarum	1	1	2	0	3	0	18	3	2	1	1	8

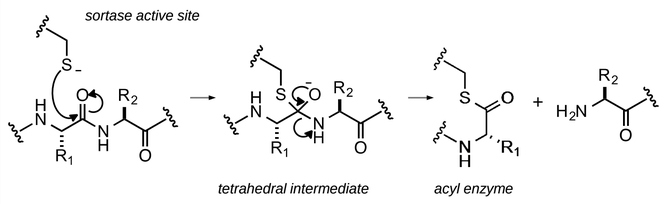
No activity observed for P, T, I, D, E, R, K, H

Reaction conditions:
25 µM sortase homolog
200 µM Abz-**LPATZ**G-K(Dnp)
10 mM H_2NOH
50 mM Tris, pH 7.5,
150 mM NaCl, 10 mM $CaCl_2$

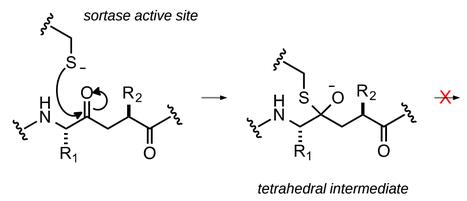
Values represent % conversion

We anticipate a better understanding of this process can be achieved by crystallizing $SrtA_{pneu}$ with a bound substrate, allowing us to evaluate key binding interactions and to compare this to other known sortase A homolog structures. Ideally, a substrate analog would be employed to allow binding to the enzyme active site without substrate cleavage. A peptidomimetic constructed with a ketomethylene linkage at the normal scissile bond in the substrate is proposed for achieving this goal.

Standard substrate cleavage by sortase A



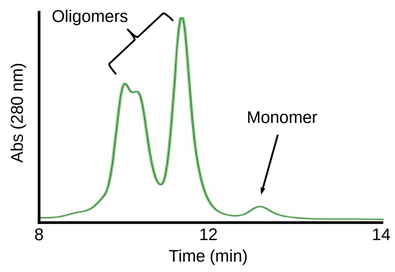
Ketomethylene analog



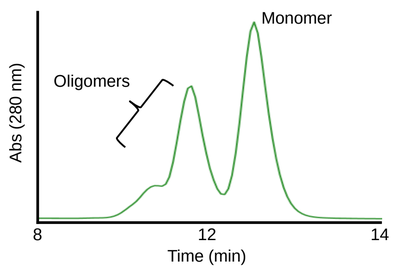
Preparation and Refolding of $SrtA_{pneu}$

- $SrtA_{pneu}$ expressed under nondenaturing conditions exhibits significant oligomerization, which decreases enzyme activity *in vitro*.
- Purification under denaturing conditions followed by refolding produces fractions with higher monomer content (right chromatogram) as compared to purification under nondenaturing conditions (left chromatogram).

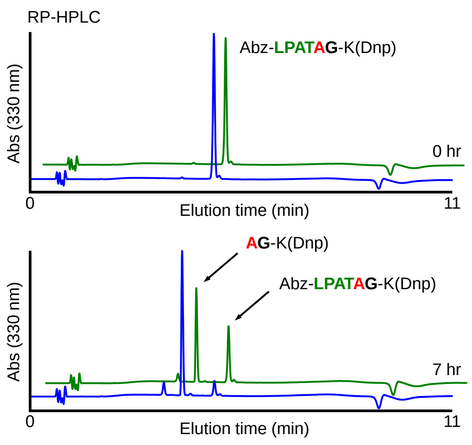
Size Exclusion (nondenaturing)

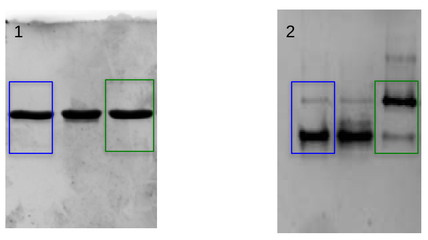


Size Exclusion (refolded)



- Monomeric $SrtA_{pneu}$ shows improved activity *in vitro*.

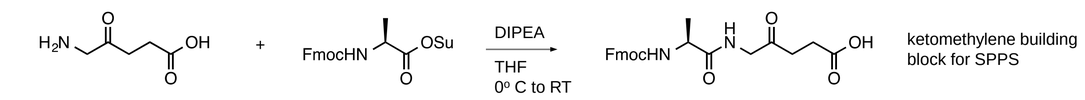




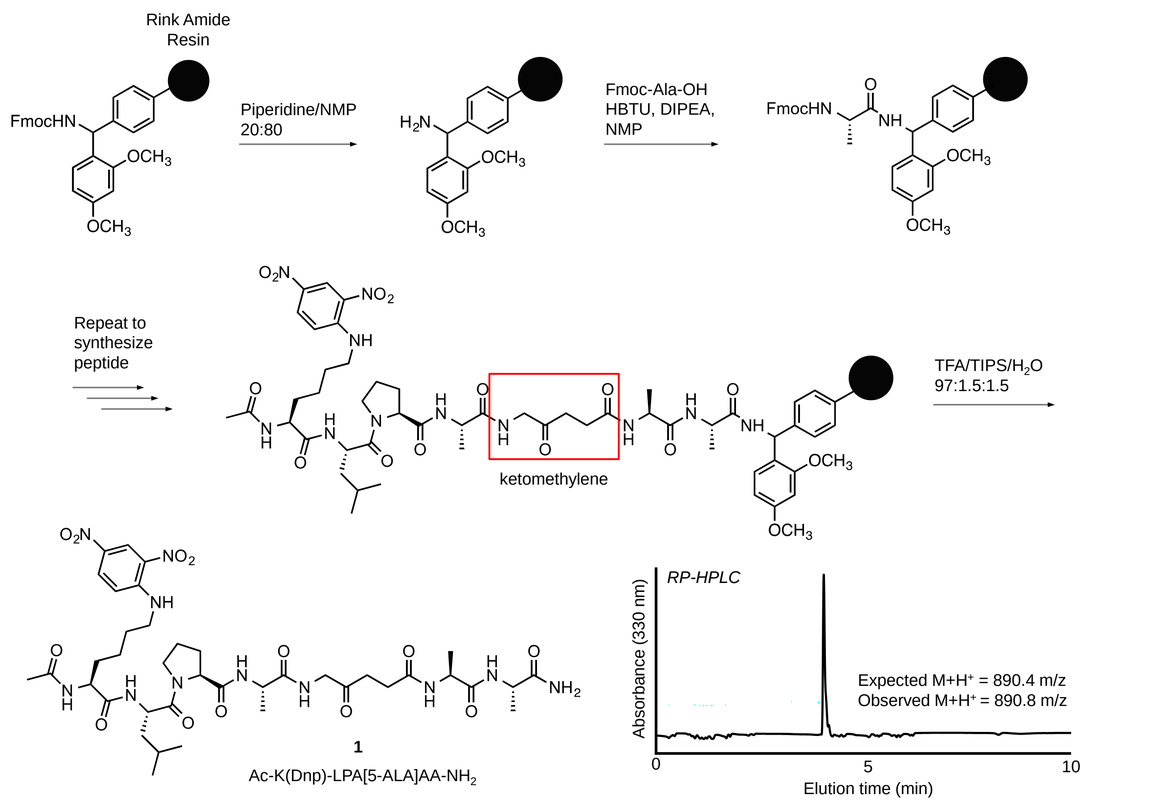
Samples from $SrtA_{pneu}$ catalyzed peptide modification reactions separated through polyacrylamide gel electrophoresis. Gel (1) contains SDS and gel (2) does not. Identical samples were loaded in the lanes of each gel. Boxed lanes correspond to samples from reactions in HPLC traces of the same color at left.

Preliminary Analog Synthesis and Testing

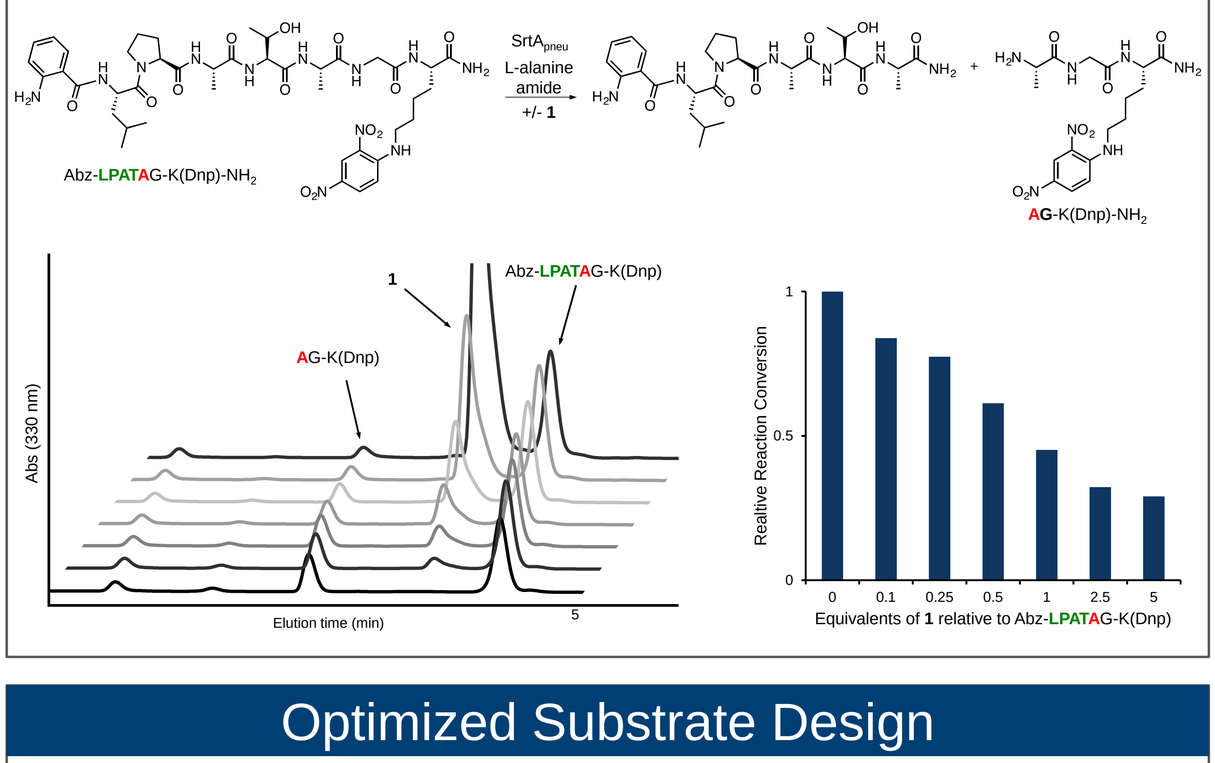
Synthesis scheme of the L-alanine-5-aminolevulinic acid pseudopeptide



Solid phase peptide synthesis (SPPS)



*Inhibition of transpeptidation using substrate analog **1***



Optimized Substrate Design

Synthesis scheme of the methylated ketomethylene isostere

Acknowledgements

This research was supported by the Research Corporation for Science Advancement and Western Washington University. The authors would like to thank Charles Wandler at WWU Scientific Technical Services for assistance with mass spectrometry, and Hla Win-Piazza for assistance with NMR spectroscopy.

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