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Substrate based peptide aldehyde inhibits bacterial type I signal peptidase

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ABSTRACT

Bacterial type I signal peptidase is a potential target for the development of novel antibacterial agents. In this study we demonstrate that a substrate based peptide aldehyde inhibits signal peptidases with a lower IC_{50} value than the lipopeptides described to date. The length of the core lipopeptide could be reduced by removing several amino acids from both termini. Conversion of this peptide to an aldehyde resulted in a molecule with an IC_{50} value of 0.09 μM when tested against *Saccharomyces aureus* SPase I, SpsB.

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Antibacterial resistance is a growing problem in the treatment of bacterial infections. To be able to face this problem we urgently need to find new targets and develop novel antibacterial drugs. Bacterial type I signal peptidase (SPase I), a key enzyme in protein secretion with the function of cleaving off the signal peptides from translocated pre-proteins, is a potential target to combat bacterial infections for several reasons. First of all, SPase I was shown to be an essential enzyme for the viability of *Escherichia coli* and *Saccharomyces aureus*.[1,2] Inhibition of SPase I activity leads to the accumulation of pre-proteins in the cell membrane and eventually to cell death.[3,4] Furthermore, type I signal peptidases utilize a unique catalytic Ser/Lys dyad mechanism[5] in both gram-positive and gram-negative bacteria, whereas eukaryotic signal peptidases contain the more classical His in place of Lys as general base.[6] Bacterial and higher order signal peptidases show low homogeneity and have significant differences in cell localization, quaternary structure and substrate specificity as well,[7] which raises the possibility of the selective inhibition of bacterial SPase I. The localization of the catalytic domain on the outer side of the membrane makes the target protein more accessible than usual cytoplasmic targets. A β-lactam type inhibitor, allyl (5*S*,6*S*)-6[(*R*)-acetoxyethyl]-penem-3-carboxylate,[8,9] arylomycin A_2[10] and novel lipoglycopeptides[11] serve as good examples for target validation, since they were found to inhibit *E. coli* SPase I and are active in vitro against *E. coli* strains. Essentiality, selectivity and accessibility are three highly important properties that make the enzyme an attractive target to develop novel antibacterial agents.

The signal peptide (Fig. 1) consists of three domains, a positively charged N-terminal domain (n-region), a hydrophobic middle domain, which contains usually 7–15 amino acids forming an α-helix (h-region) and a C-terminal domain (c-region), which is responsible for substrate recognition. Statistical analysis of amino acid sequences around the cleavage site revealed that small, neutral side chains are required in the P_1 and P_3 positions. In most cases alanines are found in these positions.[12,13] Therefore, the substrate recognition rule is often called the Ala-X-Ala (AXA) rule. This observation was later confirmed by the solution of the crystal structure of the *E. coli* SPase I LepB,[14] identifying two small hydrophobic pockets (S_1 and S_3) on the surface of the catalytic domain of the enzyme.

Since signal peptidases belong to the serine protease family, the most obvious attempt to obtain a good inhibitor was the screening of the known general serine protease inhibitors. Interestingly none of these were found to inhibit SPase I.[15,16] Moreover, the classical approach of inhibiting serine proteases with aldehydes, fluoroketones or chloroketones, based on the sequence of the Ala-X-Ala recognition motif, failed to inhibit SPase I.[17] It was believed that this was due to the unique Ser/Lys dyad protease mechanism. Selection of alternative inhibitors, such as using β-lactam compounds[8,17,18] or substrate based peptides with a proline in the P_1' position converting a substrate into an inhibitor,[19,20] was more successful. Among them, one of the most potent was a 13mer lipopeptide, Decanoyl-LTPTAKAPSKIDD-OH.[21] A 13mer is a long peptide when it comes to drug design and a decanoyl group gives the compound an amphiphilic property, which might be not very favorable for biodistribution. Therefore, we investigated the possibility that the length of the peptide can be reduced while keeping biological activity. Likewise, the role of the decanoyl group was

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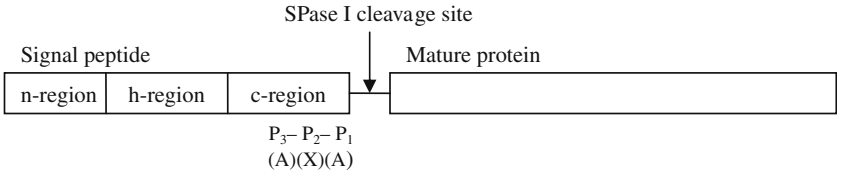

Figure 1. Schematic structure of the signal peptide. Signal peptide consists of three domains: the positively charged n-region, the hydrophobic, helical h-region and the C-terminal c-region with a β-sheet conformation. SPase I cleaves after the Ala-X-Ala recognition motif.

investigated. Furthermore, we reconsidered the original belief that SPase I cannot be inhibited by the classical serine protease type of inhibitors, that is, by substrate-based peptide aldehydes.[17] Previously observed inactivity of such compounds could be due to the fact that only short sequences, based on the Ala-X-Ala recognition motif, were tested. Here we demonstrate that it is possible to reduce the length of the selected peptide inhibitor and introduce an aldehyde group resulting in an increased potency towards its SPase I target.

The lipopeptide inhibitor of SPase I (**1**) described by Bruton and co-workers[21] was based on the consensus sequence of *S. aureus* pre-proteins. They replaced the P'_1 alanine with a proline in order to convert the substrate to an inhibitor. The N-terminal decanoyl moiety was believed to act as a membrane anchor. This inhibitor is one of the most potent published in the literature so far. As the structure of this compound is not very attractive for drug design, we decided to examine the importance of the structural elements of this inhibitor by omitting the decanoyl chain and systematically reducing the number of amino acids one by one at both termini. This approach resulted in a set of lipopeptides shown in Table 1.

Compounds were synthesized on a solid support[22] and tested in vitro on isolated signal peptidase enzymes (SpsB and LepB) and in different bacterial cell cultures. SpsB SPase I from *S. aureus* was selected because the original inhibitor was based on the consensus sequence of pre-proteins from *S. aureus* whereas LepB from *E. coli*, the best characterized SPase I until now, was selected in order to evaluate the possibility that the compound can be used as a lead for the development of antibiotics against gram-positive and gram-negative species.

The evaluation of the inhibitory activity of the synthesized compounds was done according to a continuous fluorometric assay, previously described for LepB.[23] This quantitative in vitro assay is based on the processing of an internally quenched fluorescent synthetic peptide substrate and is suitable for kinetic analysis and high-throughput screening of inhibitors (FRET-based assay). After cleavage of the peptide by the SPase, an increase of fluorescence can be measured. Inhibition of SPase I can be analyzed and quantified by measuring the processing rate of the cleavage of the internally quenched peptide substrate by SPase I in the presence and absence of the potential inhibitors. A similar FRET based assay for SpsB and LepB has been worked out for evaluation of potential inhibitors. (Smitha Rao C.V. et al., FEBS J., in press).

Testing the antimicrobial activity against *S. epidermidis*, *S. aureus* and *E. coli* was carried out according to the standard NCCLS protocol following the broth-microdilution method.[24]

The results of the in vitro tests on the isolated enzymes are summarized in Table 2. Each compound was tested at a maximum concentration of 200 μM. If inhibition did not reach 50%, the IC_{50} value was not determined (>200 μM). In these cases only the inhibitory effect at the highest concentration tested is shown. More potent compounds were further diluted and tested (in triplicate) at twelve different concentrations to determine the IC_{50} values.

Comparison of the activity of compounds **1** and **2** confirms that a long N-terminal aliphatic chain improves the efficacy of the inhibitor, at least in the enzyme test system. However, it is not clear whether this is due to better binding affinity or to a solubilization effect. If we consider the fact that our biological test system is effective only in the presence of a detergent, in our case Triton X-100, the detergent itself can be responsible for catalyzing the recognition process between the decanoylated peptide and the SPase I by bringing them in close proximity. Triton X-100 is believed to mimic the cell membrane[14] in the in vitro isolated enzyme tests, hence decanoylation might be needed in vivo as well. However, the proposed function of the decanoyl chain as a membrane anchor stays a hypothesis. Nevertheless, it is clear that it is a crucial structural element to obtain an active peptide based inhibitor.

By evaluating the lipopeptide set resulting from the deletion of N-terminal amino acids (compounds **1**, **3**, **4**, **5** and **6**) we observe a six and four fold loss of activity in the case of compound **3** and compound **4** respectively when compared to compound **1**. A similar degree of loss in activity can be observed when the activity of compound **5** is compared with the activity of compound **3** or **4**. This means that amino acids in positions P_7, P_6 and P_5, Leu, Thr and Pro in this particular case, play an important, however not crucial role in the binding of the peptide to signal peptidase. If we consider the total loss of activity in the case of P_4 deletion (compound **6**), we can conclude that this position is essential for good inhibi-

Table 1
Structure of the compounds

Code	Stucture of the peptides
1	Decanoyl-LTPTAKAPSKIDD-OH[a]
2	H-LTPTAKAPSKIDD-OH
3	Decanoyl-TPTAKAPSKIDD-OH
4	Decanoyl-PTAKAPSKIDD-OH
5	Decanoyl-TAKAPSKIDD-OH
6	Decanoyl-AKAPSKIDD-OH
7	Decanoyl-LTPTAKAPSKID-OH
8	Decanoyl-LTPTAKAPSKI-OH
9	Decanoyl-LTPTAKAPSK-OH
10	Decanoyl-LTPTAKAPS-OH

[a] Lead compound, described by Bruton and co-workers.[21]

Table 2
Results of the inhibitory activity assay for compounds **1–10**

Compounds	*S. aureus* SpsB (0.1 μM)	
	IC_{50} (μM)[a]	Inhibition (%) at 200 μM[a]
1	0.8 (±0.2)	107.1 (±0.4)
2	>200	21.6 (±0.9)
3	4.8 (±0.5)	106.2 (±0.8)
4	3.2 (±0.2)	105.8 (±2.6)
5	30.8 (±7.6)	98.0 (±0.7)
6	>200	0
7	5.0 (±1.2)	107.9 (±1.8)
8	2.6 (±0.09)	106.6 (±3.7)
9	1.0 (±0.2)	107.5 (± 1.0)
10	22.5 (±4.4)	92.9 (±5.5)

[a] Values are means of three experiments, standard deviation is given in parenthesis.

tory activity. However, this does not necessarily mean that the amino acid in this position plays a crucial role in substrate recognition. It is also possible that it plays a role as a non-specific linker between the Ala-X-Ala recognition motif and the decanoyl chain which interacts with the membrane or the micelles formed by the detergent added in the in vitro experiments. Hence, an amino acid screening at this position would help to understand the role of P_4 amino acid and could help to find better inhibitors as well.

Evaluation of the other set of molecules (compounds **7**–**10**) resulting from elimination of amino acids from the C-terminal, showed a lower effect on inhibition of SPase I. However, it is worth noting that elimination of P'_3–P'_6 amino acids resulted in a twenty fold drop in activity. Interestingly, deletion of P'_5 and P'_4 resulted in a two and five fold increase of activity, respectively, compared to compound **7**. Compound **9** shows the same activity as the reference compound **1**. Compounds **1**–**10** showed no inhibition at the highest concentration tested against *E. coli* LepB.

These results indicate that the minimal length of an inhibitory lipopeptide consists of six amino acids of the composition decanoyl-TAKAPS-OH. This peptide sequence can serve as a scaffold for further modifications, such as modification at the positions P_4, P_2 and P'_2 for optimizing the sequence and incorporating unnatural amino acids as well, in order to make it less prone to degradation by digestive enzymes.

We further investigated whether this peptide, with the C-terminal carboxylic acid (at the cleavage site) converted to an aldehyde, would be a potent SPase I inhibitor. Proline and serine in P'_1 and P'_2 positions were eliminated from the sequence since the aldehyde function should be introduced at the position of the scissile bond. We extended the N-terminal part with one amino acid, namely proline at the P_5 position, in order to compensate for potential loss of activity resulted by P'_1 and P'_2 deletion. However, the specific extent of this compensation by P_5 extension was not determined. To avoid intramolecular interaction between the lysine side chain amino function and the C-terminal aldehyde function of the peptide we decided to replace P_2 lysine with asparagine, shown to be a reasonable alternative[21] in this position. This decanoyl-PTANA-aldehyde[25,26] (**11**) (Fig. 2) was tested in the same test system as compounds **1**–**10** and was found to inhibit SpsB with an IC_{50} value of 0.09 μM (Table 3) making it a better inhibitor than the reference compound **1**. Whereas compounds **1**–**10** showed very limited effect on LepB, the peptide aldehyde (**11**) was found to inhibit LepB with an IC_{50} value of 13.4 μM. These data indicate that a substrate based peptide aldehyde can be a better inhibitor than compounds, obtained by the replacement of P'_1 amino acid with proline. Compounds **1** and **11** were tested against *S. aureus*, *S. epidermidis* and *E. coli* cell cultures at different concentrations. The reference compound (**1**) showed no antibacterial activity on the three types of bacterial cultures, whereas compound **11** inhibited cell growth in a dose dependent manner in the case of *S. aureus* and *S. epidermidis* with MIC_{50} values of 125–500 μg/ml, respectively, which is too high to consider the compound for further development. *E. coli* growth was not inhibited by compound **11** at the highest concentration tested (500 μg/ml) This is probably due to the lower affinity of the inhibitor towards the target enzyme LepB and the extra outer membrane of the gram-negative *E. coli* bacteria, making the target less accessible.

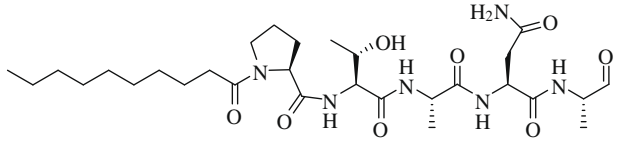

Figure 2. Structure of compound **11** (Decanoyl-PTANA-aldehyde).

Table 3
Results of the inhibitory activity assay and antibacterial activity for compound **11**

Compd	IC_{50} (μM)[a]		MIC (μg/ml)		
	S. aureus SpsB	*E. coli* LepB	*S. aureus*	*S. epidermidis*	*E. coli*
11	0.09 (±0.03)	13.4 (±1.3)	125	500	>500

[a] Values are means of three experiments, standard deviation is given in parenthesis.

Summarizing the results we can conclude that the N-terminal decanoyl chain of the reference lipopeptide **1** is essential for its activity, whereas deletion of amino acids from the P′ side of the scissile bond seems to have less effect on activity. Deletion of the N-terminal amino acids decreases the activity fivefold each on average, except that deletion of P_6 results in no change of activity. The P_4 amino acid seems to have an essential role in the inhibitory effect. Altogether, this study showed that some of the amino acids of the 13-mer peptide are not necessary for its activity and we could identify an essential peptide core, that is, TAKAPS-OH which consists of six amino acids. Although, the decanoyl derivative of this peptide is not very active, it is a much smaller compound and can serve as a starting point for developing peptidomimetics or other types of signal peptidase inhibitors. To test this hypothesis, we converted the optimized peptide to a peptide aldehyde and this compound was able to inhibit bacterial signal peptidase I. This peptide aldehyde showed inhibitory activity ten times better than the reference compound and it was not only able to inhibit SpsB from the gram-positive *S. aureus*, but also LepB from the gram-negative *E. coli*. Furthermore, this inhibitor showed a moderate effect on microbial growth of *S. aureus*. Further research will be concentrated on the optimization of its structure by investigating the peptide sequence, incorporating unnatural amino acids and introducing peptidomimetic scaffolds.

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