Peptidoglycan Hot Paper

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Muropeptides in *Pseudomonas aeruginosa* and their Role as Elicitors of β-Lactam-Antibiotic Resistance

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Abstract: *Muropeptides are a group of bacterial natural products generated from the cell wall in the course of its turnover. These compounds are cell-wall recycling intermediates and are also involved in signaling within the bacterium. However, the identity of these signaling molecules remains elusive. The identification and characterization of 20 muropeptides from Pseudomonas aeruginosa is described. The least abundant of these metabolites is present at 100 and the most abundant at 55,000 molecules per bacterium. Analysis of these muropeptides under conditions of induction of resistance to a β-lactam antibiotic identified two signaling muropeptides (N-acetylglucosamine-1,6-anhydro-N-acetylmuramyl pentapeptide and 1,6-anhydro-N-acetylmuramyl pentapeptide). Authentic synthetic samples of these metabolites were shown to activate expression of β-lactamase in the absence of any β-lactam antibiotic, thus indicating that they serve as chemical signals in this complex biochemical pathway.*

The cell wall (also known as the sacculus) is a complex macromolecular polymer that encases the bacterium. Its major constituent is comprised of repeating *N*-acetylglucosamine (NAG)–*N*-acetylmuramic acid (NAM), with a pentapeptide stem attached to the NAM unit.[1] The cell wall is critical for survival of the bacterium, hence the cell wall and its biosynthetic machinery are targets for antibiotics.[2] The cell wall is synthesized through polymerization of lipid II[3,4] to give the NAG–NAM backbone, which is subsequently crosslinked to the neighboring strand through the peptide stem (Figure 1). In parallel, degradative processes of the assembled cell wall also take place.[4,5] An important event in this degradation is mediated by a family of enzymes called lytic transglycosylases (LTs), the reactions of which generate a series of natural products referred to collectively as muropeptides.

The reactions of all eight *Escherichia coli* LTs have been studied in vitro.[6–8] These enzymes generate a transient oxocarbenium species (**1**) at the muramyl moiety of the peptidoglycan, which results in cleavage of the β-1,4-glycosidic bond between a NAM and a NAG moiety (Figure 1), giving rise to the 1,6-anhydromuramyl moiety (**2** and **3**). Some LTs perform this reaction at the ends of the peptidoglycan, the so called exolytic reaction, to give NAG-anhNAM disaccharides (**2**). Others perform the reaction in the middle of the peptidoglycan, the endolytic reaction, which gives rise to a longer backbone for the sugar (**3**). These released muropeptides are translocated to the cytoplasm by the permease AmpG (and possibly by AmpP in *Pseudomonas aeruginosa*).[9] Once in the cytoplasm, the muropeptides enter the cell-wall recycling process to regenerate lipid II.[4,8,10] Alternatively, other muropeptides are involved in signaling functions, leading to disparate responses such as antibiotic resistance, virulence, and inflammation.[11,12] Their role in resistance to β-lactam antibiotics involves binding to the gene regulator AmpR, which induces transcription of the gene *ampC* for the AmpC β-lactamase from Gram-negative

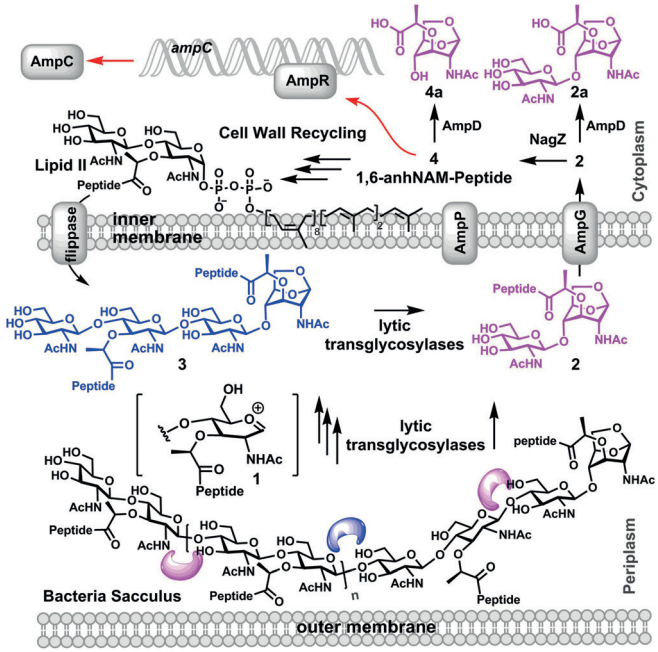

Figure 1. The reactions of LTs produce muropeptides containing the 1,6-anhydromuramyl moiety, which are translocated by the permease AmpG (and AmpP in *P. aeruginosa*). In normal growth of the bacteria, **4** would initiate recycling events to regenerate lipid II. However, as an offshoot of the recycling events, accumulation of **4** induces β-lactamase (AmpC) expression (indicated by red arrows).

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bacteria (Figure 1).[13] β-Lactamase hydrolyzes β-lactam antibiotics, which inhibit the action of pencillin-binding proteins by mimicking the structure of the terminal D-Ala-D-Ala in the stem peptides of the peptidoglycan.

The processes that muropeptides mediate are not fully understood owing to impediments such as the minute quantities present, rapid metabolic flux, and complexity of the structures. Once the structures are elucidated, they need to be prepared in the laboratory for validation of the assigned structure and for conducting biochemical studies. Herein, we report the identification, characterization, and quantification of several muropeptides from the periplasm of *P. aeruginosa*, an opportunistic human pathogen.

Sample preparation is important, since dilution of the minute quantities present and contamination could confound analysis. Initially, osmotic shock was used for liberation of the periplasmic contents, which contains the muropeptides. This should separate the periplasmic and cytoplasmic metabolites before attempts at isolation of the muropeptides. Unfortunately, 10–30 % cytoplasmic contamination was noted in these samples. The results were also not reproducible, and muropeptides could merely be identified near the detection limit of 0.4 pmol by our instrumentation.

The muropeptides that enter the cytoplasm via the permease AmpG (or AmpP) are expected to undergo rapid metabolic flux (Figure 1). This effect was confirmed by preparing spheroplasts of *P. aeruginosa* PAO1. The cytoplasmic content from the lysed spheroplasts were analyzed by LC–MS for muropeptides. None could be detected, thus suggesting that the cytoplasmic muropeptides were rapidly metabolized to lipid II, with concentrations below our detection limit. Hence, the whole bacterium was grown and lysed, an approach that proved to be reliable and reproducible. The muropeptides that were generated under these conditions could only have come from the intact periplasm in the whole bacterium. After sample preparation (see the Supporting Information), LC–MS analyses were performed for the detection and identification of muropeptides.

The muropeptide content of the whole cells of *P. aeruginosa* PAO1 was compared in the absence and presence of half of the minimal-inhibitory concentration (MIC) of the antibiotic cefoxitin, a β-lactam that interferes with cell-wall synthesis.[14] Cefoxitin at sub-MIC levels efficiently activates the expression of β-lactamase, thereby leading to antibiotic resistance in *P. aeruginosa*.[14] This is believed to be mediated by a messenger function of a muropetide.[12,15,16] Hence, one or more of the muropeptides listed in Figure 2 A is expected to serve as the signaling molecule for antibiotic resistance.

As shown in Figure 2 A, compounds are numbered according to the nature of the sugar, **2** for NAG-1,6-anhNAM and **4** for 1,6-anhNAM. For the peptide component, **a** has no peptide, **b**, **c**, **d**, and **e** carry di-, tri-, tetra- and pentapeptides, respectively (the full pentapeptide is L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala; bottom right of Figure 2 A). Compounds **3** are (NAG-NAM-peptide)$_n$-NAG-1,6-anhNAM-peptide and compounds **5** are for cross-linked species. For example, compound **5dd** indicates cross-linked peptide between two tetrapeptides. Compounds with reducing-end sugars, which lack the 1,6-anhNAM, were also

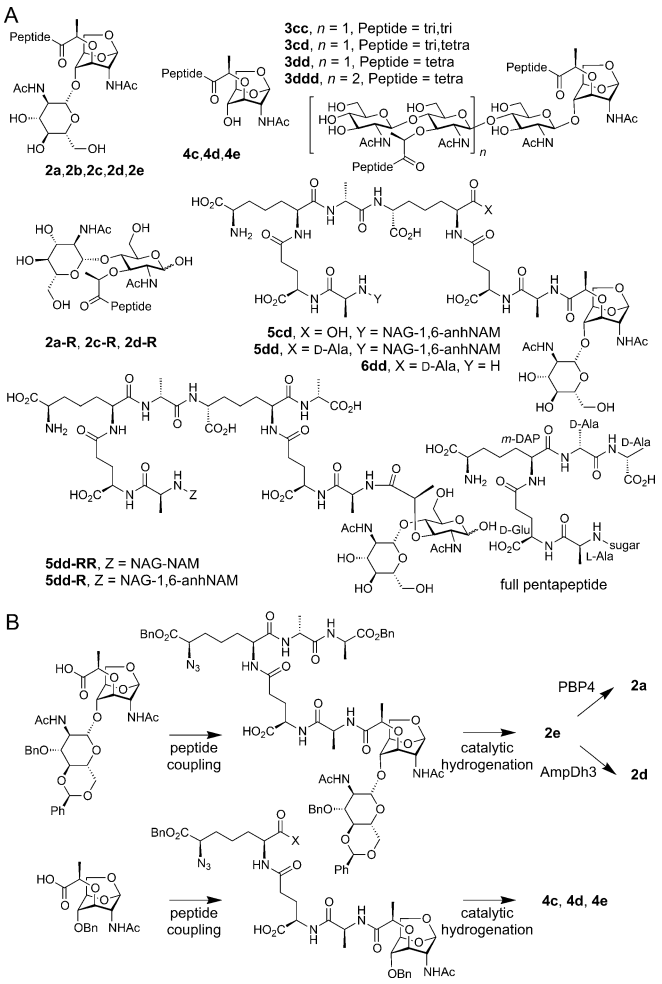

Figure 2. A) Chemical structures of detected muropeptides. B) The chemoenzymatic syntheses of six muropeptides.

detected as minor components and are designated by the letter **R**.

Figure 3 A shows the LC–MS total-ion chromatogram (TIC) of the pseudomonal sacculus turnover products in the presence of the purified *E. coli* LT MltA. In contrast to this typical in vitro analysis with the isolated sacculus, samples from the whole cell did not reveal any discernable muropeptides (Figure 3 B). This necessitated the preparation of authentic standards for comparison to the LC–MS extracted-ion chromatograms (EICs) of individual metabolite (Figures 3 C–F).

Four muropeptides, **2e**,[17] **4c**, **4d**, and **4e**,[18,19] were synthesized. A few of these authentic samples were also converted into new species by known enzymatic reactions (Figure 2 B and Figure S1 in the Supporting Information). For example, **2e** was converted into **2d** by penicillin-binding protein 4 (PBP4),[15] and **2e** into **2a** by AmpDh3,[19] both purified recombinant enzymes from *P. aeruginosa*. Figures 3 C–F show EICs of the detected metabolites **2a**, **2d**, **2e**, **4c**, and **4d**, and comparison to the authentic standards (Figure 3 G). Further analysis was performed through comparison of the MS and MS/MS with authentic samples, as exemplified in Figure S1. For structure assignment of metab-

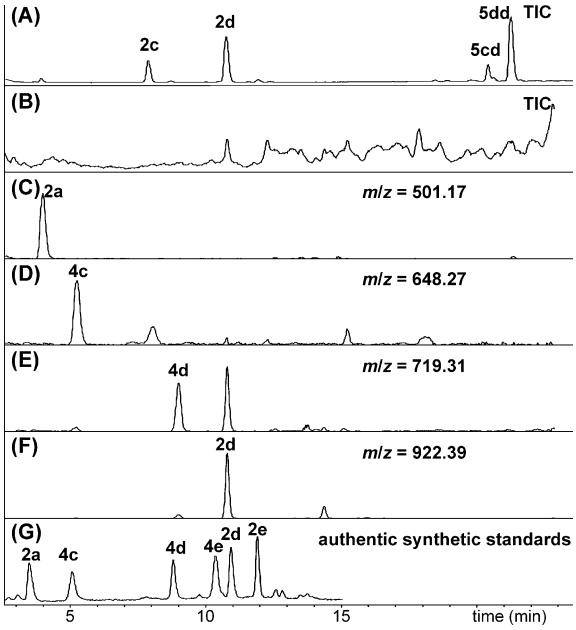

Figure 3. Analysis of *P. aeruginosa* muropeptides. The LC–MS TICs of sacculus digested by MltA (A) and whole-cell analysis (B). EICs of **2a** (C), **4c** (D), **4d** (E), and **2d** (F) from whole-cell samples, and the TIC of authentic synthetic standards mixed together (G).

olites for which authentic standards were not available, the method that was developed previously by our laboratory to analyze turnover products of sacculus by LTs and PBP4 was used.[6,15]

Quantification was done by integrating peak areas from EICs of the corresponding *m*/*z* values of the individual muropeptide. This was converted into concentration by using standard curves generated with the authentic **2e**. The concentration was then converted into numbers of molecules (of each compound) per bacterium (Table 1 in the Supporting Information). Standard curves for **2e**, **4c**, **4d**, **4e**, and **7** (β-methoxy-NAG-NAM(pentapeptide)-NAG-NAM (pentapeptide))[20] were very similar, within 7% variation of each other (Chart S1 and Figure S2). Our collection of synthetic standards covers distinctive chemical structures of more than 95% of the detected muropeptides. **2e** was chosen as a representative synthetic standard for quantification.

The most abundant muropeptide in the wild-type PAO1 strain is NAG-1,6-anhNAM-tetrapeptide (**2d**). The di-, tri-, and pentapeptide variants (**2b**, **2c**, and **2e**) are also found, along with **2a** (with no peptide). These are reaction products of LTs, mostly from exolytic activity. The discovery of compounds with the core 1,6-anhNAM-peptides (**4c** and **4d**) suggests the existence of the *N*-acetylglucosaminidase activity in *P. aeruginosa*. The presence of such an enzyme (FlgJ) in the periplasm was recently documented in *Salmonella enterica*.[21] This activity in *P. aeruginosa* might be mediated by PA1085, which shows an identity of 31% and a similarity of 46% at the amino acid level to FlgJ from *S. enterica* (Figure S3).[21] To our knowledge, this is the first documentation of a periplasmic *N*-acetylglucosaminidase reaction product in *P. aeruginosa*.[21] Oligomeric sugars (up to hexamers) with tetrapeptide (**3dd** and **3ddd**) or a mix of

Table 1: Detected muropeptides from whole-cell analysis (in molecules per bacterium $\times 10^4$).[a]

Muropeptide	Wild-type	Induced[b]	*p*-value[c]
NAG-1,6-anhNAM			
2a	1.5 ± 0.07	0.3 ± 0.01	0.01*
2b	0.2 ± 0.01	0.08 ± 0.01	0.004*
2c	0.8 ± 0.05	0.3 ± 0.02	0.02*
2d	5.5 ± 0.3	1.0 ± 0.3	0.002*
2e	0.01 ± 0.005	0.1 ± 0.02	0.04*
1,6-anhNAM			
4c	0.3 ± 0.06	0.1 ± 0.003	0.0003*
4d	0.4 ± 0.01	0.07 ± 0.003	0.01*
4e	N.D.[d]	0.08 ± 0.01	0.04*
(NAG-NAM)$_n$-NAG-1,6-anhNAM			
3cc	0.1 ± 0.01	0.02 ± 0.006	0.003*
3cd	0.2 ± 0.02	0.03 ± 0.007	0.03*
3dd	0.9 ± 0.06	0.09 ± 0.03	0.002*
3ddd	0.1 ± 0.02	0.01 ± 0.003	0.049*
NAG-1,6-anhNAM-crosslinked-1,6-anhNAM-NAG			
5cd	0.03 ± 0.004	0.02 ± 0.001	0.2
5dd	0.3 ± 0.01	0.07 ± 0.007	0.001*
6dd	0.04 ± 0.001	0.02 ± 0.004	0.07
Reduced end			
2a-R	0.07 ± 0.01	N.D.[d]	0.02*
2c-R	0.07 ± 0.03	0.02 ± 0.002	0.11
2d-R	0.5 ± 0.1	0.1 ± 0.03	0.06
5dd-RR	0.1 ± 0.002	0.01 ± 0.002	0.0002*
5dd-R	0.02 ± 0.002	0.009 ± 0.002	0.047*
Total[e]	11.1 ± 0.8	2.4 ± 0.5	

[a] Average of two runs. [b] PAO1 was exposed to cefoxitin at 512 μg mL^{-1}. [c] *p*-values from Students *t*-test. :* significant difference ($p < 0.05$) between the wild-type and induced samples. [d] Not detected. [e] Total detected muropeptides.

tetra and tripeptide (**3cd** and **3cc**) were also found. These are products of endolytic reactions of LTs.[6]

Cross-linked muropeptides such as **5cd**, **5dd**, and **6dd** were also found. As minor components, muropeptides containing a sugar with a reducing end (**2a-R**, **2c-R**, **2d-R**, **5dd-RR**, and **5dd-R**) were also detected. This indicates that the reactive oxocarbenium species partitions between entrapment of either the internal C_6-hydroxy or a water molecule, or that there exists a yet-to-be identified hydrolytic glycosidase in this organism. The ratio of the two types of products (non-reducing to reducing) is approximately 14:1.

The same sample preparation and analyses were carried out with *P. aeruginosa* PAO1 exposed to the cell-wall-active antibiotic cefoxitin at half of the MIC (512 μg mL^{-1}), in other words, at a non-lethal concentration.[14] Exposure to the antibiotic alters the pool of muropeptides, and one or more is understood to enter the cytoplasm via AmpG (or AmpP) permease and upregulate the production of β-lactamase, the antibiotic-resistance determinant.[9,12] The induction of resistance was confirmed by β-lactamase assay with nitrocefin.

The same number of bacteria and the same conditions were used in both cases; hence, the values of the two columns of Table 1 can be compared to each other. The analysis showed that the total muropeptide (molecules/bacterium) was significantly reduced (*p*-value < 0.05 by Student's *t*-test) in the antibiotic-induced vs. the uninduced bacteria (24,000 vs. 111,000; Table 1). The most abundant muropeptide in the

uninduced sample was **2d** (NAG-1,6-anhNAM-tetrapeptide). Muropeptide **2e** (NAG-1,6-anhNAM-pentapeptide) was enriched 46-fold upon antibiotic induction (1000 in 24,000 molecules vs. 100 in 111,000 molecules). This indicates that the β-lactam antibiotic inhibits the targeted PBP, lack of the activity of which leaves its peptidoglycan substrate in the sacculus intact. This observation in living bacteria agrees with the finding of an in vitro sacculus analysis of the induced *P. aeruginosa*.[15,16] Compound **4e** was detected only in the induced sample. This observation suggests that as the concentration of **2e** increases upon induction, the compound is likely broken down by the aforementioned *N*-acetylglucosaminidase to produce **4e**. Other than **2e** and **4e**, the rest of the muropeptides detected in the induced sample were similar to those in the uninduced cells. It is not immediately obvious why the total quantity of muropeptides is lower in the induced sample (one fifth of the uninduced). This likely reflects altered regulation of cell-wall modifications of the bacterium in the presence of sub-lethal concentrations of the antibiotic.

The obvious question now becomes whether exogenously added authentic muropeptides **2e** or **4e** could cause induction of antibiotic resistance in the absence of antibiotic. We investigated this first with the wild-type *P. aeruginosa* PAO1 strain. Addition of muropeptide **2e** or **4e** at upwards of 500 $\mu g\,mL^{-1}$ failed to induce β-lactamase expression (Table 2). Going with the premise that the outer membrane of Gram-negative bacteria is a formidable barrier to the penetration of most small molecules into the periplasm, we procured a mutant strain defective in the outer membrane. The strain *P. aeruginosa* Z61 has the full complement of the genes necessary for the induction of β-lactamases, but it expresses a mutant version of the β-lactamase with diminished activity.[22] Nonetheless, when using the nitrocefin assay, we observed a 4.7-fold increase in β-lactamase induction at a quarter-MIC level of cefoxitin. The same experiment performed with the bacteria exposed to 100 $\mu g\,mL^{-1}$ muropeptides **2e** or **4e** resulted in 1.4- or 1.7-fold induction, respectively (Table S1). This is a large excess of these compounds, but we used them this way because we expected that the exogenously added compounds would undergo degradation by periplasmic enzymes. Hence, the two metabolites produced in response to the exposure of bacteria to the antibiotic inducer cefoxitin collectively account for most of the induction observed by cefoxitin. Compound **2a** and **4a** (metabolites without a peptide stem; products of the reaction of AmpD) were used as negative controls. As expected, under the same conditions, induction was not observed. The activity of AmpD (Figure 1) is at the crossroads of induction of resistance versus cell-wall recycling (reversal of induction).

This study reports the nature and quantities of 20 muropeptides from *P. aeruginosa*. The levels of muropeptide **2e** increased 46-fold upon exposure of the bacteria to sub-MIC levels of the β-lactamase inducer cefoxitin. We also observed muropeptide **4e** only in the induced cells. This study shows that authentic synthetic samples of muropeptides **2e** and **4e** could serve as inducers of β-lactam-antibiotic resistance in the absence of antibiotic. These experiments clearly domonstrate that muropeptides **2e** and **4e** are chemical elicitors of the induction of antibiotic resistance.

Table 2: Induction of β-lactamase activity by three inducers.[a]

Inducer	Wild-type (PAO1) Conc. [$\mu g\,mL^{-1}$]	β-lactamase induction[c]		Mutant (Z61)[b] Conc. [$\mu g\,mL^{-1}$]	β-lactamase induction[c]	
Cefoxitin[d]	512	136[e]	1555/11.4	0.0156	4.7[e]	4.6/0.98
2e	500	1.0	19.1/19.1	100	1.4[e]	27.5/20.0
4e	500	1.0	19.1/19.1	100	1.7[e]	5.5/3.3
2a[d]		–[f]		10[g]	1.0	4.1/4.0
4a[d]		–[f]		100	1.0	3.6/3.5

[a] Average of two runs. [b] The strain is defective in its outer membrane. [c] The number (the left column) is calculated from lactamase activity in the presence of an inducer divided by that without an inducer (the right column) under the same conditions. The enzyme activity was expressed as nmol of nitrocefin hydrolyzed per min per mg of protein for wild-type and pmol/min/mg for mutant. [d] Cefoxitin and compounds **2a** and **4a** were used as positive and negative controls for induction, respectively. [e] β-Lactamse activities were significantly different ($p < 0.05$) between induced and uninduced samples. [f] Not measured. [g] Owing to limited supply of compound, we could only assess the effect at the lower concentration.

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