DOI: 10.1002/cbic.200800678

Specific Labeling of Peptidoglycan Precursors as a Tool for Bacterial Cell Wall Studies

Vincent van Dam, Nick Olrichs, and Eefjan Breukink*[a]

Because of its importance for bacterial cell survival, the bacterial cell wall is an attractive target for new antibiotics in a time of great demand for new antibiotic compounds. Therefore, more knowledge about the diverse processes related to bacterial cell wall synthesis is needed. The cell wall is located on the exterior of the cell and consists mainly of peptidoglycan, a large macromolecule built up from a three-dimensional network of aminosugar strands interlinked with peptide bridges. The subunits of peptidoglycan are synthesized inside the cell before they are transported to the exterior in order to be incorporated into the growing peptidoglycan. The high flexibility of the cell wall synthesis machinery towards unnatural derivatives of these subunits enables research on the bacterial cell wall using labeled compounds. This review highlights the high potential of labeled cell wall precursors in various areas of cell wall research. Labeled precursors can be used in investigating direct cell wall–antibiotic interactions and in cell wall synthesis and localization studies. Moreover, these compounds can provide a powerful tool in the elucidation of the cell wall proteome, the "wallosome," and thus, might provide new targets for antibiotics.

1. Introduction

One of the most essential structures a bacterium possesses is its cell wall. It forms a suit of armour and allows the bacterium to withstand high differences in osmotic pressures between that of its cytoplasm and that of the environment. It also helps the cell maintain its shape and provides an anchor for proteins[1] and other cell envelope constituents such as Braun's lipoprotein[2] and polysaccharides.[3] The cell wall plays a role in all segments of the bacterial growth cycle, and its construction requires a complex and elaborate ensemble of biochemical and assembly processes; these properties make it an important structure. Because of its importance for bacterial cell survival and its high accessibility, the cell wall is a target for antibiotics. This is illustrated by the fact that many of the known antibiotics, such as penicillin,[4] vancomycin[5] and bacitracin,[6,7] attack the bacteria at the level of cell wall synthesis. However, bacterial resistance against antibiotics is causing severe problems in healthcare. Resistant strains have even been reported against vancomycin, which used to be the antibiotic of last resort to treat infections of the infamous methicillin-resistant *Staphylococcus aureus* (MRSA) bug.[8] Hence, the demand for new antibiotics is now bigger than ever. In the search for these antibiotics, the bacterial cell wall and its whole synthesis pathway are perfect leads, because the cell wall still provides many uncharacterized areas that might serve as a target for antibiotic agents.

This review focuses on biochemical methods that can be applied to unravel unknown areas and mechanisms involved with the bacterial cell wall that might serve as a target for antibiotic agents, and in particular, their use in the field of proteomics to characterize the cell wall proteome, the so-called "wallosome."

2. Peptidoglycan Synthesis

The bacterial cell wall mainly consists of peptidoglycan, which is a huge macromolecule consisting of a multilayered network of linear glycan chains interlinked with each other by short peptide moieties. The glycan chain is composed of alternating β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues (Figure 1.) The carboxyl group of each MurNAc unit is substituted by a small peptide that contains amino acids in the D-configuration, which is a typical characteristic of peptidoglycan. By crosslinking to each other, the peptide moieties on the MurNAc residues form peptide bridges interlinking the glycan strands.[9–11] Since the peptides are arranged helically along the glycan stands, it is possible to form crosslinks in all directions.[10] In this way a multilayered, three-dimensional network is formed[12]

The synthesis of peptidoglycan occurs in different stages. It starts in the cytoplasm where the aminosugar subunits uridine-5′-diphosphate-GlcNAc (UDP-GlcNAc) and UDP-MurNAc-pentapeptide are synthesized. In *Escherichia coli,* UDP-MurNAc-pentapeptide is synthesized in six steps, starting with the transfer of an enolpyruvate (PEP) to UDP-GlcNAc by MurA. Subsequently, MurB catalyses the reduction of the PEP moiety to D-lactate; this yields UDP-*N*-acetylmuramate. After this, four ATP-dependent aminoligases—MurC, MurD, MurE and MurF—catalyze the stepwise addition of the pentapeptide chain to

[a] *V. van Dam,[+] N. Olrichs,[+] Prof. E. Breukink*
Chemical Biology and Organic Chemistry, Bijvoet Center for Biomolecular Research and Institute of Biomembranes
Utrecht University
Padualaan 8, 3584 CH Utrecht (The Netherlands)
Fax: (+31) 30-253-3969
E-mail: e.j.breukink@uu.nl
[+] *These authors contributed equally to this work.*

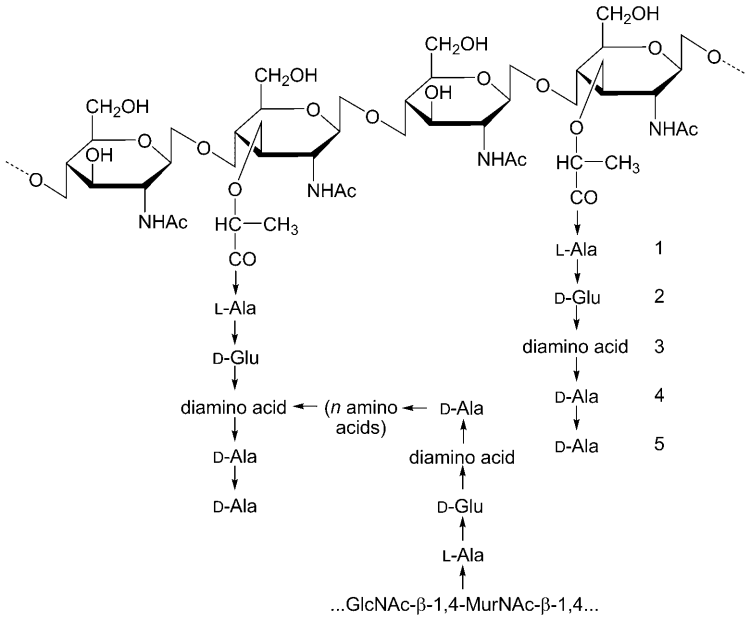

Figure 1. A general schematic representation of two peptidoglycan subunits. Amino acid positions of the pentapeptide are numbered **1**–**5**. In addition to the composition of the pentapeptide, the length (*n*) and composition of the interpeptide bridge also varies among the bacterial species (for a review see ref. [44]). Figure 1 is adapted from ref. [101].

the D-lactate. This results in the complete UDP-MurNAc-pentapeptide molecule.[13–18]

The following steps still occur on the cytoplasmic side, but the location is now on the membrane surface.[19] This amounts to the transfer of the phospho-MurNAc-pentapeptide of UDP-MurNAc-pentapeptide to a special membrane-bound lipid acceptor, undecaprenyl-phosphate, yielding lipid I. This step is catalyzed by the integral membrane protein MraY (Figure 2, step B).

In the second membrane-bound step, UDP-GlcNAc is coupled to lipid I by the glycosyltransferase MurG resulting in lipid II (Figure 2C). Thus, lipid II contains a complete monomer unit of the peptidoglycan layer of *E. coli*, GlcNAc-MurNAc-pentapeptide. In *E. coli*, MurG is the last protein known to be in contact with lipid II on the inside of the bacterial cytoplasmic membrane, so the next step in the cycle involves the transfer of lipid II across the cytoplasmic membrane to the exterior in order to get incorporated into the growing peptidoglycan chain (Figure 2D). The mechanism by which this membrane transport occurs has been elusive so far. However, recent studies claim the involvement of the putative flippase MviN in this process.[20,21]

The following steps are also membrane-bound but take place on the exterior side of the cytoplasmic membrane and involve the activity of glycosyltransferases and transpeptidases. The glycosyltransferases catalyze the formation of the linear glycan chains that contain repeats of the alternating aminosugars. Transpeptidases catalyze the formation of peptide cross-links between the glycan strands and the incorporation of the glycan strands into the existing peptidoglycan (Figure 2E). Although monofunctional transglycosylases and transpeptidases exist, the main peptidoglycan synthesizing activity stems from bifunctional proteins. These are often referred to as penicillin binding proteins (PBPs) because antibiotics of the β-lactam class specifically inhibit them. *E. coli* possesses twelve PBPs of which PBP1a and PBP1b are the major bifunctional transpeptidase-transglycosylases.[22]

After the GlcNAc-MurNAc-pentapeptide moiety is released by the action of PBP1b,[23,24] a free undecaprenylpyrophosphate molecule is generated. The membrane-bound undecaprenyl carrier is transported back to the cytoplasmic side of the membrane by an unknown mechanism (Figure 2E). After the removal of one phosphate group from the undecaprenolpyrophosphate[25–27] (Figure 2A), the molecule can be reused in a new cycle of peptidoglycan synthesis.

3. Peptidoglycan Recycling

Not only is the undecaprenyl carrier molecule recycled, but *E. coli* also possesses an efficient recycling pathway for peptidoglycan building blocks (for a review, see ref. [28]). This recycling pathway includes the breakdown of the cell wall by the action of lytic transglycosylases and endopeptidases (Figure 3A). These enzymes release monomeric anhydro-MurNAc-L-Ala-D-Glu-*m*-DAP-D-Ala from the peptidoglycan into the periplasm. Most often these muropeptides are degraded to tripeptide derivatives by the action of L,D-carboxypeptidases. The muropeptides can enter two different recycling pathways. The

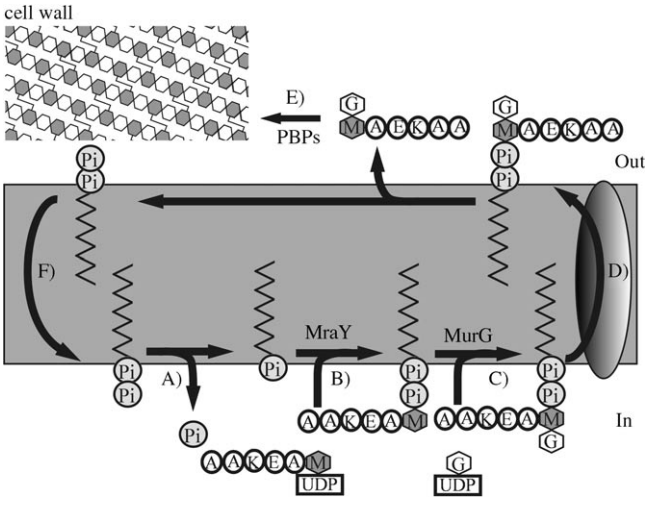

Figure 2. Membrane-bound cycle of peptidoglycan synthesis. Starting from the cytoplasmic side; after the A) dephosphorylation of undecaprenylpyrophosphate, B) UDP-MurNAc is attached to undecaprenylphosphate by MraY, forming lipid I. C)After this, MurG catalyzes the addition of UDP-GlcNAc, which results in lipid II, carrying one complete peptidoglycan subunit. D) Subsequently, lipid II is transported across the membrane through an unknown mechanism. E) Thereafter, the peptidoglycan subunit is cleaved of the undecaprenyl carrier and incorporated into the growing peptidoglycan chain. F) Finally, the undecaprenylpyrophospate is transported back to the cytoplasmic side, by a mechanism that is also unknown, allowing for the beginning of a new cycle. For a review, see ref. [19].

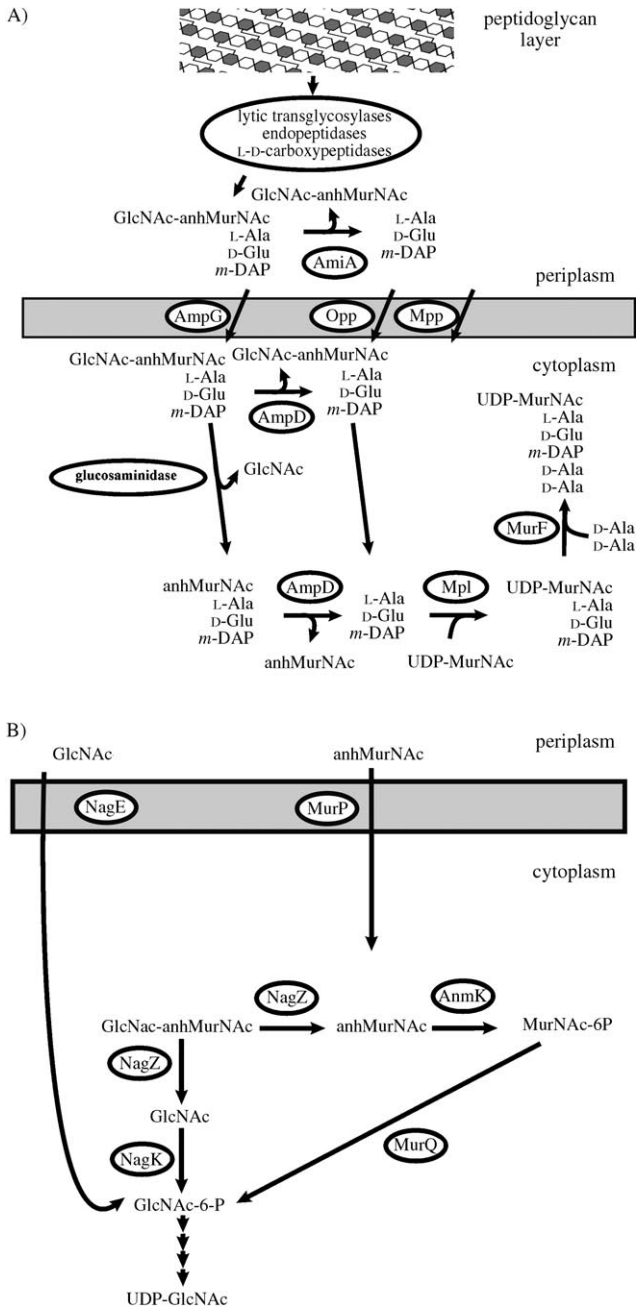

Figure 3. Recycling of peptidoglycan in *E. coli*. A) Recycling of peptidoglycan peptides. B) Recycling of peptidoglycan aminosugars.

first possibility involves a periplasmic amidase (AmiA) which cleaves the muropeptide into a disaccharide and a tripeptide.[29] Subsequently, the tripeptide can be transported across the cytoplasmic membrane by the general oligopeptide transport system Opp[30] or the specific murein peptide permease Mpp.[31] The other possibility is that the intact muropeptides are taken up by AmpG[32,33] and subsequently degraded by the specific amidase activity of AmpD.[34,35] This yields anhydro-MurNAc (anhMurNac), GlcNAc and free tripeptides. The latter are coupled to UDP-MurNAc by the specific tripeptide ligase Mpl, resulting in UDP-MurNAc-tripeptide.[36] This molecule is the normal substrate of MurF and the peptide has now re-entered the cell wall synthesis pathway.

The aminosugars of the peptidoglycan are also recycled (Figure 3 B). NagZ cleaves the GlcNAc-anhMurNAc to release GlcNAc and anhMurNAc.[37] GlcNAc is phosphorylated by the specific kinase activity of NagK, thereby returning to the pathway for UDP-GlcNAc synthesis.[38] The anhMurNAc molecule is first phosphorylated by AnmK.[39] Subsequently, MurNAc-6P is converted to GlcNAc-P by the action of the MurQ etherase.[40] AnhMurNac is also directly taken up into the cell by the action of MurP.[40] Likewise, GlcNAc can be imported by NagE.[41] Although peptidoglycan recycling also occurs in Gram-negative species other than *E. coli*, Gram-positive bacteria lose about 50% of their peptidoglycan every cell cycle, and no mechanism of cell wall recycling is known in Gram-positive bacteria.[42]

4. Variations in Peptidoglycan Composition

Although peptidoglycan always consists of glycan strands interlinked by peptide bridges, the composition of the aminosugars and peptides shows great variation among the various bacterial species.[11,43,44] In a few bacteria, the glycan chain displays minor variations including *O*- or *N*-acetylation, which probably occur at late stages of cell wall maturation.[11]

The peptide moiety is usually synthesized as a pentapeptide; however, due to peptidoglycan maturation, sometimes tri-, tetra- or hexapeptides are also found.[12,45–48] Not only can the length of the peptides vary, but also the amino acids of the peptide moiety show variation among different bacterial species. Variations occur at all the peptide positions but especially the amino acid at position three is prone to variation (Figure 1). At this position a diamino acid is usually found, and the most common are *meso*-diaminopimelic acid (DAP) and L-lysine. DAP is present in virtually all *E. coli* and other Gram-negative bacteria but can also be found in other species like some lactobacilli, clostridia, bacilli, corynebacteria and propionibacteria.[11] By contrast, L-lysine is rarely observed in Gram-negative species.[49] Less frequent variants at position three also occur among the bacterial kingdom. The amino acids at the other positions can also show variations; however, these are not common.[11]

5. High Flexibility of the Cell Wall Synthesis Machinery

In addition to the high level of variation in the composition of peptidoglycan among the diverse bacterial species, the cell wall synthesis machinery also shows a high tolerance towards unnatural substrates. MraY and MurG display broad substrate specificity since these proteins proved capable of lipid II synthesis utilizing (fluorescently) labeled substrates as well as prenyl carriers with prenyl chain lengths varying from 2–25 isoprene units.[50,51] Recent studies showed that only a limited number of conserved residues were essential for the activity of MurG[52] or MraY.[53] Both MurG and MraY are present in virtually all species of the bacterial kingdom, and homologues of MraY

even exist in eukaryotic systems. In addition, the MGD protein of *Spinacia oleracea*, which catalyses the transfer of a galactosyl group from UDP-galactose onto a diacylglycerol acceptor, shows sequence similarity with *E. coli* MurG.[54] Also, in eukaryotic systems such as *Arabidopsis thaliana* and the moss *Physcomitrella patens*, homologues of *mray*, various *mur* genes and even genes encoding for PBPs can be found.[55] Bacteria can also show tolerance towards unnatural amino acids in their cell wall. For instance, mutants of *E. coli* lacking a diaminopimelate epimerase were found to have a larger amount of LL-diaminopimelic acid (LL-DAP) than *meso*-DAP incorporated into their peptidoglycan layer.[56,57] Moreover, in vivo experiments showed that DAP could be totally replaced by *meso*-lanthionine or L-*allo*-cystationine in the peptidoglycan of *E. coli*.[58]

6. Studies on Bacterial Cell Wall Inhibitors Using Labeled PG Derivatives

The high flexibility of the bacterial cell wall synthesis machinery makes peptidoglycan subunits ideal tools in cell wall research because it opens up the possibility to employ precursors modified by a wide variety of labels. Many pioneering studies on the biosynthesis of peptidoglycan involved the use of radiolabeled precursors,[59–61] and they were involved in the elucidation of the mode of action of different antibiotics[62,63] and the discovery of the recycling pathway[30]. Beside their usefulness for analyses of bacterial cell wall metabolism, radiolabeled peptidoglycan and its precursors are also of great interest for investigations of host-bacteria interactions. For example, the injection of [^{14}C]-DAP-labeled peptidoglycan fragments in *Drosophila* allowed for the demonstration of the role of the amidase activity of the peptidoglycan-recognition protein (PGRP)-LB in the response to bacterial infection and innate immunity mechanisms in flies.[64] Although radiolabeled cell wall precursors remain a valuable tool in cell wall studies, the high flexibility of the bacterial cell wall synthesis machinery opened up the possibility to employ precursors modified by a wide variety of labels. This drastically expanded the possibilities to study cell wall synthesis and inhibitors thereof in great detail.

The molecular mechanism of action of different classes of antibiotics and the various interactions they form with cell wall precursors has been extensively studied with labeled versions of these precursors. One of the earliest studies using labeled peptidoglycan derivatives employed UDP-MurNAc-pentapeptide that was chemically modified at its lysine residue with the spin-label Tempyo (2,2,5,5-tetramethyl-*N*-oxylpyrroline-3-carbonyl).[65,66] A spin label provides information about its microenvironment and mobility, and the labeled UDP-MurNAc-pentapeptide was used to study the interactions with the antibiotics vancomycin and ristocetin during various stages of in vitro peptidoglycan synthesis.

In another study, the interactions of lipid II, labeled with pyrene at its lysine, with the peptide antibiotic nisin were investigated.[67,68] This antibiotic kills bacteria through so-called targeted pore formation.[69] By the analysis of the characteristic excimer fluorescence of pyrene, lipid II was shown to be an integral part of the nisin pore. The size, stoichiometry, and stability of the nisin-lipid II pore complex were elucidated by means of this technology.[70]

Furthermore, the molecular basis for the inhibition of MraY from *E. coli* by two classes of antibiotics, the mureidomycins and the liposidomycins was assayed with a fluorescence-based enzyme assay.[71,72] UDP-MurNAc-pentapeptide, dansylated at its DAP, was used as a substrate. This allowed for the kinetic characterization of mureidomycin A, tunicamycin and liposidomycin B. This method was subsequently expanded to a high-throughput assay to screen for inhibitors of MraY.[73] A high-throughput approach was also reported to identify glycosyltransferase inhibitors. This involved the displacement of a UDP-GlcNAc derivative, fluorescently modified at its *N*-acetyl group, from the glycosyl donor binding site, and this screen was successfully applied to *E. coli* MurG.[74,75]

Aside from being a valuable tool for interaction studies and inhibitor screenings, chemically modified precursors themselves could be promising candidates for a new type of antibiotic. Fluorinated carbohydrate derivatives are extensively applied in medical sciences as substrate mimics for the inhibition of enzymatic processes, because most enzymes are not able to differentiate between the fluorinated and original compounds. Lipid I analogues, fluorinated at the C-4 position of the MurNAc residue, were shown to be potent inhibitors of MurG,[76] and when Gram-positive bacteria were incubated in the presence of UDP-MurNAc pentapeptide fluorinated at the position C-4, significant inhibition of growth was observed.[77] As muramic acid is specifically present in all bacteria, a fluorinated muramic acid derivative could be an ideal candidate.

7. Use of Labeled Peptidoglycan Derivatives in PG Synthesis Studies

In order to study the reactions catalyzed by enzymes in the lipid-linked cycle of bacterial peptidoglycan biosynthesis, fluorescently labeled cell wall precursors have been extensively exploited. As the quantum yield of certain fluorophores increases significantly in hydrophobic surroundings, enzymatic reactions involving changes in the hydrophobic environment of specific substrates are ideally suited for fluorescence assays.

The investigation of enzymes utilizing lipid I and lipid II has long been hindered by the difficulty of acquiring these substrates in useful quantities from natural sources. In an effort to overcome this problem, several groups have now reported the chemical synthesis of lipid II.[51,78,79] Alternatively, a semisynthetic approach was developed to synthesize large quantities of lipid II and variants.[50] This involves supplying bacterial membrane preparations rich in MraY and MurG with polyisoprenyl phosphates, UDP-MurNAc-pentapeptide and UDP-GlcNAc. Fluorescent lipid II was produced with UDP-MurNAc-pentapeptide modified at its lysine residue with a fluorescent label.

The positioning of labels on the pentapeptide moiety is of importance for the study of enzymes catalyzing the formation of peptide crossbridges. For instance, UDP-MurNAc-pentapeptide was fluorescently derivatized at various positions and used for enzymological studies on *E. coli* membrane-bound enzymes.[80] This strategy was based on the enzymatic prepara-

tion of analogues containing D-cysteine instead of D-alanine at positions four and five, which subsequently were fluorescently labeled with pyrene maleimide.

Furthermore, an assay based on fluorescence resonance energy transfer (FRET) was developed for MurG.[81] In this way, the kinetic parameters for the enzymatic processing by MurG were determined with UDP-*N*-acetylglucosamine, fluorescently labeled at the C-6 position with indole-3-acetic acid and lipid I labeled with dansyl at the DAP residue. In line with this, fluorescent assays were developed to study the polymerization of a fluorescently labeled lipid II derivative by *E. coli* PBP1b[82] and the glycosyltransferase domains of *Thermotoga maritima* PBP1a[83] and *Streptococcus pneumoniae* PBP2a.[84]

Labeled cell wall precursor derivatives have also been utilized as an effective tool to study surface display on living bacteria. UDP-MurNAc-pentapeptide was modified with fluorescein or a ketone group. Bacteria were cultured in the presence of the modified precursors, and the groups were displayed on the bacterial surface through cell wall biosynthesis.[85–87] For incorporation of the precursors in Gram-negative bacteria, additional EDTA treatment was required to permeabilize the outer membrane. When *Lactobacilli* were treated with the ketone-modified precursor, oligomannose was coupled with the ketone moiety on the bacterial surface through an aminooxyl linker. The adhesion of these cells onto a lectin-containing surface increased significantly compared to that of native bacteria. Since bacterial adhesion is an important event in the infection of host cells and in the interaction between bacteria, controlling the adhesion properties of the bacterial surface could provide benefits to the development of novel bacterial drugs.[88] Furthermore, this bacterial engineering technique is potentially applicable to various types of ligand–receptor interactions.

Another important application of labeling the cell wall is in the research on peptidoglycan metabolism throughout the bacterial cell cycle. Peptidoglycan segregation was studied with the ability of *E. coli* to incorporate D-cysteine through an unclear mechanism of periplasmic amino acid exchange into its peptidoglycan.[89] Following the biotinylation of the cysteine thiol groups, the distribution of modified peptidoglycan in purified peptidoglycan sacculi could be traced and visualized by immunodetection with fluorescence and electron microscopy techniques. In this way, it was shown that at the initiation of cell division, there is a localized activation of peptidoglycan synthesis at the potential division sites, while the diffuse insertion of precursors occurs along the cylindrical part of the cell surface during cell elongation. Moreover, the peptidoglycan in polar caps was shown to be metabolically inert in this study. This method was further applied to reveal mechanisms of bacterial morphogenesis.[90–92]

Recently, a new method was developed to label peptidoglycan components in vivo, which can be used for multiple areas of cell wall synthesis research.[93] This method involves the incorporation of labeled peptidoglycan precursors into the cell wall by means of the cell wall recycling pathway. In this way, it was shown that *E. coli* is capable of importing exogenous murein tripeptide, fluorescently labeled at its lysine with *N*-7-nitro-2,1,3-benzoxadiazol-4-yl (AeK-NBD), into the cytoplasm where the ligase Mpl introduces it into the peptidoglycan biosynthesis route (Figure 4). Mpl has been shown to have broad

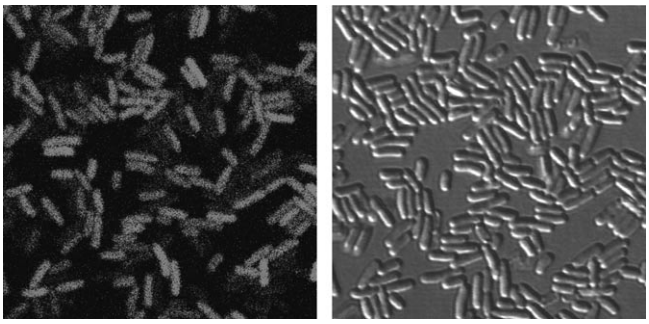

Figure 4. Wild-type *E. coli* W3899 cells incubated with AeK-NBD, analyzed by fluorescence microscopy. Image of the cells in fluorescence (left) and differential interference contrast mode (right).

substrate specificity, accepting tri- to pentapeptides containing either DAP or lysine.[94] Control experiments showed that the labels had entered the cell wall synthesis pathway as both fluorescently labeled lipid II could be extracted from treated bacteria and fluorescent labels were shown to be present in isolated cell walls. This method of incorporation of reporter groups in the cell wall of bacteria does not require the (mis)treatment of the cells in any way. Using the fluorescent approach, peptidoglycan synthesis and recycling in relation to cell growth and division was studied.[93]

8. The use of Labeled Peptidoglycan Derivatives in the Search for the lipid II Flippase

Labeled peptidoglycan precursors were also applied in the quest for what can be considered the holy grail of cell wall research, the transport mechanism of lipid II. In order to get incorporated into the growing peptidoglycan chain, lipid II has to be transported across the bilayer. The mechanism by which this occurs largely remains a black box in bacterial cell wall synthesis. Fluorescence spectrometry studies that used lipid II labeled with a dansyl group showed that the rate of unassisted lipid II movement was not sufficient to support peptidoglycan synthesis in bacteria.[95] Recently, the transbilayer movement of lipid II was analyzed through a fluorescence assay for the determination of phospholipid membrane asymmetry.[96] For this purpose, the fluorescent label 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) was attached to the lysine residue of the MurNAc-pentapeptide moiety of lipid II. The sensitivity of the NBD label on lipid II for reduction by dithionite (this leads to the nonfluorescent 7-amino-2,1,3-benzoxadiazol-4-yl, ABD) enables determination of the topology of lipid II. In this way, it was confirmed that lipid II does not flip-flop spontaneously across the bilayer of model membranes.[96] Additionally, when membrane-spanning peptides, known to induce flip-flop of some phospholipids,[97] were incorporated in model membranes, no significant transmembrane movement of the precursor could be detect-

ed.[96,97] However, lipid II translocation could be demonstrated in *E. coli* inner membrane vesicles by using NBD-labeled precursors.[96] These observations provided strong evidence that a protein is needed for the translocation of lipid II across the bilayer.

The studies described above provided the parameters that allow lipid II translocation in isolated bacterial membrane systems. This paved the way for the identification of proteins involved in this process through the use of lipid II carrying photoactivatable crosslinkers.[98] For this purpose, UDP-MurNAc-pentapeptide was labeled with *N*-hydroxysuccinimidyl-4-azidosalicyclic acid (NHS-ASA). The resulting UDP-MurNAc-pentapeptide-ASA was labeled with ^{125}I to enable the specific detection of crosslinked proteins (Figure 5 A). This radiolabeled ASA precursor was used as a substrate for lipid II synthesis in right-side-out vesicle preparations (RSOs) of *E. coli* and resulted in a set of crosslinked proteins (Figure 5 B). The labeling of proteins obtained in this way is specific, since the band pattern of radiolabeled proteins in lane 2 is different from the Coomassie pattern in lane 4. A combination of two-dimensional gel analysis and mass spectrometry was used to identify proteins that were in contact with lipid II under these conditions and resulted in an interesting set of candidates. These involved components of the multidrug efflux pump AcrAB-TolC. Strikingly, many components of the protein translocon were also found (SecY, SecA and SecD) including YidC, which is suggested to bind to the sec translocon in the membrane.[99] The role of these proteins in lipid II translocation remains unclear as of yet.

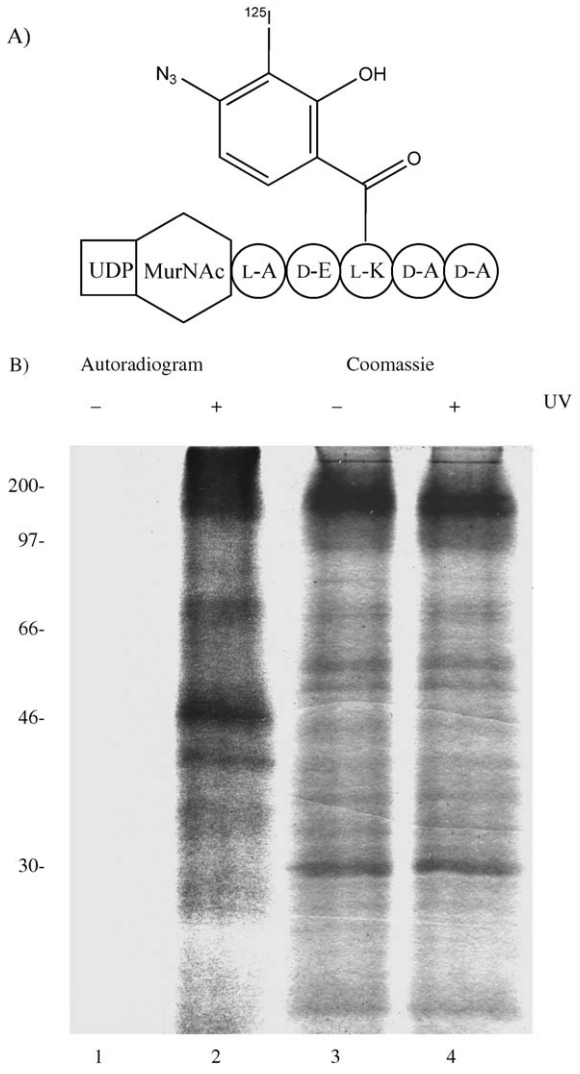

Figure 5. Radiolabeled UDP-MurNAc-pentapeptide-ASA can be used as a tool for crosslinking studies. A) Schematic representation of iodinated UDP-MurNAc-pentapeptide-ASA. The first alanine (L-A) attached to the sugar moiety and the lysine (L-K) are in the L configuration, glutamic acid (D-E) and the two ultimate alanines (D-A) are in the D configuration, with the iodinated ASA crosslinker chemically coupled to the ε-NH_2 of the L-lysine residue at position three. B) Using UDP-MurNAc-pentapeptide-[^{125}I]ASA in a crosslinking experiment results in a specific set of crosslinked proteins. UDP-MurNAc-pentapeptide-[^{125}I]ASA and UDP-GlcNAc were incorporated into vesicles before UV activation. The left panel represents the autoradiogram of a gel loaded with vesicles that were not illuminated (lane 1) or illuminated with UV light (lane 2). The right panel shows the Coomassie-stained protein pattern of the same gel (lanes 3 and 4).

9. Prospects

Exploiting the high tolerance of the bacterial cell wall synthesis machinery with labeled peptidoglycan derivatives can open a panorama of possibilities in cell wall related research. These compounds can be used to study interactions between antibiotics and peptidoglycan components. Secondly, our novel method of labeling the cell wall of *E. coli* with reporter groups in vivo paves the way for a proteomics approach. Using photo-crosslinking approaches in combination with click chemistry,[100] proteins interacting with the cell wall or its precursors can be identified, allowing for the clear elucidation of the "wallosome." The identification of the components can result in new targets for antibiotics. Furthermore, site-specific information on lipid II binding sites in penicillin-binding proteins can be obtained by photo-crosslinking in combination with mass spectrometry techniques. Finally, the use of labeled peptidoglycan derivatives can give more insight into the spatial and temporal arrangement of peptidoglycan synthesis.

Acknowledgements

This work was supported by the European commission within the EUR-INTAFAR network.

Keywords: antibiotics · bacteria · lipid II · peptides · peptidoglycans

[1] W. W. Navarre, O. Schneewind, *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 174.
[2] V. Braun, U. Sieglin, *Eur. J. Biochem.* **1970**, *13*, 336.
[3] F. C. Neuhaus, J. Baddiley, *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 686.
[4] J. T. Park, J. L. Strominger, *Science* **1957**, *125*, 99.
[5] H. R. Perkins, *Biochem. J.* **1969**, *111*, 195.
[6] D. R. Storm, J. L. Strominger, *J. Biol. Chem.* **1973**, *248*, 3940.
[7] D. R. Storm, J. L. Strominger, *J. Biol. Chem.* **1974**, *249*, 1823.
[8] B. E. Murray, *N. Engl. J. Med.* **2000**, *342*, 710.
[9] H. J. Rogers, H. R. Perkins, J. B. Ward, *Microbial Cell Walls and Membranes*, Chapman and Hall, London, **1980**.
[10] J. V. Holtje, *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 181.
[11] K. H. Schleifer, O. Kandler, *Bacteriol. Rev.* **1972**, *36*, 407.
[12] W. Vollmer, D. Blanot, M. A. de Pedro, *FEMS Microbiol. Rev.* **2008**, *32*, 149.

[13] E. D. Brown, E. I. Vivas, C. T. Walsh, R. Kolter, *J. Bacteriol.* **1995**, *177*, 4194.
[14] D. Mengin-Lecreulx, B. Flouret, J. van Heijenoort, *J. Bacteriol.* **1982**, *151*, 1109.
[15] Y. Mizuno, M. Yaegashi, E. Ito, *J. Biochem.* **1973**, *74*, 525.
[16] M. J. Pucci, L. F. Discotto, T. J. Dougherty, *J. Bacteriol.* **1992**, *174*, 1690.
[17] F. Pratviel-Sosa, D. Mengin-Lecreulx, J. van Heijenoort, *Eur. J. Biochem.* **1991**, *202*, 1169.
[18] H. Barreteau, A. Kovac, A. Boniface, M. Sova, S. Gobec, D. Blanot, *FEMS Microbiol. Rev.* **2008**, *32*, 168.
[19] A. Bouhss, A. E. Trunkfield, T. D. Bugg, D. Mengin-Lecreulx, *FEMS Microbiol. Rev.* **2008**, *32*, 208.
[20] N. Ruiz, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 15553.
[21] A. Inoue, Y. Murata, H. Takahashi, N. Tsuji, S. Fujisaki, J. I. Kato, *J. Bacteriol.* **2008**; in press.
[22] E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala, P. Charlier, *FEMS Microbiol. Rev.* **2008**, *32*, 234.
[23] F. Ishino, K. Mitsui, S. Tamaki, M. Matsuhashi, *Biochem. Biophys. Res. Commun.* **1980**, *97*, 287.
[24] J. Nakagawa, S. Tamaki, S. Tomioka, M. Matsuhashi, *J. Biol. Chem.* **1984**, *259*, 13937.
[25] M. El Ghachi, A. Bouhss, D. Blanot, D. Mengin-Lecreulx, *J. Biol. Chem.* **2004**, *279*, 30106.
[26] M. El Ghachi, A. Derbise, A. Bouhss, D. Mengin-Lecreulx, *J. Biol. Chem.* **2005**, *280*, 18689.
[27] L. D. Tatar, C. L. Marolda, A. N. Polischuk, D. van Leeuwen, M. A. Valvano, *Microbiology* **2007**, *153*, 2518.
[28] J. T. Park, T. Uehara, *Microbiol. Mol. Biol. Rev.* **2008**, *72*, 211.
[29] J. van Heijenoort, C. Parquet, B. Flouret, Y. van Heijenoort, *Eur. J. Biochem.* **1975**, *58*, 611.
[30] E. W. Goodell, C. F. Higgins, *J. Bacteriol.* **1987**, *169*, 3861.
[31] J. T. Park, D. Raychaudhuri, H. Li, S. Normark, D. Mengin-Lecreulx, *J. Bacteriol.* **1998**, *180*, 1215.
[32] S. Lindquist, K. Weston-Hafer, H. Schmidt, C. Pul, G. Korfmann, J. Erickson, C. Sanders, H. H. Martin, S. Normark, *Mol. Microbiol.* **1993**, *9*, 703.
[33] J. T. Park, *J. Bacteriol.* **1993**, *175*, 7.
[34] J. V. Holtje, U. Kopp, A. Ursinus, B. Wiedemann, *FEMS Microbiol. Lett.* **1994**, *122*, 159.
[35] C. Jacobs, B. Joris, M. Jamin, K. Klarsov, J. Van Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, J. M. Frere, *Mol. Microbiol.* **1995**, *15*, 553.
[36] D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, *J. Bacteriol.* **1996**, *178*, 5347.
[37] Q. Cheng, H. Li, K. Merdek, J. T. Park, *J. Bacteriol.* **2000**, *182*, 4836.
[38] T. Uehara, J. T. Park, *J. Bacteriol.* **2004**, *186*, 7273.
[39] T. Uehara, K. Suefuji, N. Valbuena, B. Meehan, M. Donegan, J. T. Park, *J. Bacteriol.* **2005**, *187*, 3643.
[40] T. Uehara, K. Suefuji, T. Jaeger, C. Mayer, J. T. Park, *J. Bacteriol.* **2006**, *188*, 1660.
[41] J. W. Lengeler, K. Jahreis, U. F. Wehmeier, *Biochim. Biophys. Acta Bioenerg.* **1994**, *1188*, 1.
[42] J. T. Park, *Mol. Microbiol.* **1995**, *17*, 421.
[43] W. Vollmer, *FEMS Microbiol. Rev.* **2008**, *32*, 287.
[44] J. M. Ghuysen, *Bacteriol. Rev.* **1968**, *32*, 425.
[45] K. Izaki, J. L. Strominger, *J. Biol. Chem.* **1968**, *243*, 3193.
[46] R. Plapp, J. L. Strominger, *J. Biol. Chem.* **1970**, *245*, 3667.
[47] W. P. Hammes, O. Kandler, *Eur. J. Biochem.* **1976**, *70*, 97.
[48] A. G. Pisabarro, R. Prats, D. Vaquez, A. Rodriguez-Tebar, *J. Bacteriol.* **1986**, *168*, 199.
[49] A. Boniface, A. Bouhss, D. Mengin-Lecreulx, D. Blanot, *J. Biol. Chem.* **2006**, *281*, 15680.
[50] E. Breukink, H. E. van Heusden, P. J. Vollmerhaus, E. Swiezewska, L. Brunner, S. Walker, A. J. Heck, B. de Kruijff, *J. Biol. Chem.* **2003**, *278*, 19898.
[51] X. Y. Ye, M. C. Lo, L. Brunner, D. Walker, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2001**, *123*, 3155.
[52] M. Crouvoisier, G. Auger, D. Blanot, D. Mengin-Lecreulx, *Biochimie* **2007**, *89*, 1498.
[53] B. Al-Dabbagh, X. Henry, M. El Ghachi, G. Auger, D. Blanot, C. Parquet, D. Mengin-Lecreulx, A. Bouhss, *Biochemistry* **2008**, *47*, 8919.
[54] C. Botte, C. Jeanneau, L. Snajdrova, O. Bastien, A. Imberty, C. Breton, E. Marechal, *J. Biol. Chem.* **2005**, *280*, 34691.
[55] M. Machida, K. Takechi, H. Sato, S. J. Chung, H. Kuroiwa, S. Takio, M. Seki, K. Shinozaki, T. Fujita, M. Hasebe, H. Takano, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 6753.
[56] D. Mengin-Lecreulx, C. Michaud, C. Richaud, D. Blanot, J. van Heijenoort, *J. Bacteriol.* **1988**, *170*, 2031.
[57] C. Richaud, W. Higgins, D. Mengin-Lecreulx, P. Stragier, *J. Bacteriol.* **1987**, *169*, 1454.
[58] C. Richaud, D. Mengin-Lecreulx, S. Pochet, E. J. Johnson, G. N. Cohen, P. Marliere, *J. Biol. Chem.* **1993**, *268*, 26827.
[59] J. S. Anderson, M. Matsuhashi, M. A. Haskin, J. L. Strominger, *Proc. Natl. Acad. Sci. USA* **1965**, *53*, 881.
[60] W. P. Hammes, F. C. Neuhaus, *J. Biol. Chem.* **1974**, *249*, 3140.
[61] Y. Higashi, J. L. Strominger, C. C. Sweeley, *Proc. Natl. Acad. Sci. USA* **1967**, *57*, 1878.
[62] G. Siewert, J. L. Strominger, *Proc. Natl. Acad. Sci. USA* **1967**, *57*, 767.
[63] J. B. Ward, *FEBS Lett.* **1977**, *78*, 151.
[64] A. Zaidman-Remy, M. Herve, M. Poidevin, S. Pili-Floury, M. S. Kim, D. Blanot, B. H. Oh, R. Ueda, D. Mengin-Lecreulx, B. Lemaitre, *Immunity* **2006**, *24*, 463.
[65] L. S. Johnston, F. C. Neuhaus, *Biochemistry* **1975**, *14*, 2754.
[66] L. S. Johnston, F. C. Neuhaus, *Biochemistry* **1977**, *16*, 1251.
[67] H. E. Hasper, N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff, E. Breukink, *Science* **2006**, *313*, 1636.
[68] E. Breukink, B. de Kruijff, *Nat. Rev. Drug Discovery* **2006**, *5*, 321.
[69] E. Breukink, I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. Sahl, B. de Kruijff, *Science* **1999**, *286*, 2361.
[70] H. E. Hasper, B. de Kruijff, E. Breukink, *Biochemistry* **2004**, *43*, 11567.
[71] P. E. Brandish, M. K. Burnham, J. T. Lonsdale, R. Southgate, M. Inukai, T. D. Bugg, *J. Biol. Chem.* **1996**, *271*, 7609.
[72] P. E. Brandish, K. I. Kimura, M. Inukai, R. Southgate, J. T. Lonsdale, T. D. Bugg, *Antimicrob. Agents Chemother.* **1996**, *40*, 1640.
[73] T. Stachyra, C. Dini, P. Ferrari, A. Bouhss, J. van Heijenoort, D. Mengin-Lecreulx, D. Blanot, J. Biton, D. Le Beller, *Antimicrob. Agents Chemother.* **2004**, *48*, 897.
[74] J. S. Helm, Y. Hu, L. Chen, B. Gross, S. Walker, *J. Am. Chem. Soc.* **2003**, *125*, 11168.
[75] Y. Hu, J. S. Helm, L. Chen, C. Ginsberg, B. Gross, B. Kraybill, K. Tiyanont, X. Fang, T. Wu, S. Walker, *Chem. Biol.* **2004**, *11*, 703.
[76] L. Chen, H. Men, S. Ha, X. Y. Ye, L. Brunner, Y. Hu, S. Walker, *Biochemistry* **2002**, *41*, 6824.
[77] T. Ueda, F. Feng, R. Sadamoto, K. Niikura, K. Monde, S. Nishimura, *Org. Lett.* **2004**, *6*, 1753.
[78] B. Schwartz, J. A. Markwalder, Y. Wang, *J. Am. Chem. Soc.* **2001**, *123*, 11638.
[79] M. S. VanNieuwenhze, S. C. Mauldin, M. Zia-Ebrahimi, B. E. Winger, W. J. Hornback, S. L. Saha, J. A. Aikins, L. C. Blaszczak, *J. Am. Chem. Soc.* **2002**, *124*, 3656.
[80] J. A. Schouten, S. Bagga, A. J. Lloyd, G. de Pascale, C. G. Dowson, D. I. Roper, T. D. Bugg, *Mol. Biosyst.* **2006**, *2*, 484.
[81] J. J. Li, T. D. Bugg, *Chem. Commun.* **2004**, 182.
[82] B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang, R. L. Stein, *Biochemistry* **2002**, *41*, 12552.
[83] J. Offant, F. Michoux, A. Dermiaux, J. Biton, Y. Bourne, *Biochim. Biophys. Acta Proteins Proteomics* **2006**, *1764*, 1036.
[84] A. M. Di Guilmi, A. Dessen, O. Dideberg, T. Vernet, *J. Bacteriol.* **2003**, *185*, 4418.
[85] R. Sadamoto, K. Niikura, P. S. Sears, H. Liu, C. H. Wong, A. Suksomcheep, F. Tomita, K. Monde, S. Nishimura, *J. Am. Chem. Soc.* **2002**, *124*, 9018.
[86] R. Sadamoto, K. Niikura, T. Ueda, K. Monde, N. Fukuhara, S. Nishimura, *J. Am. Chem. Soc.* **2004**, *126*, 3755.
[87] R. Sadamoto, K. Niikura, K. Monde, S. Nishimura, *Methods Enzymol.* **2003**, *362*, 273.
[88] N. Sharon, I. Ofek, *Glycoconjugate J.* **2000**, *17*, 659.
[89] M. A. de Pedro, J. C. Quintela, J. V. Holtje, H. Schwarz, *J. Bacteriol.* **1997**, *179*, 2823.
[90] M. A. de Pedro, W. D. Donachie, J. V. Holtje, H. Schwarz, *J. Bacteriol.* **2001**, *183*, 4115.

[91] M. A. de Pedro, K. D. Young, J. V. Holtje, H. Schwarz, *J. Bacteriol.* **2003**, *185*, 1147.
[92] M. Aaron, G. Charbon, H. Lam, H. Schwarz, W. Vollmer, C. Jacobs-Wagner, *Mol. Microbiol.* **2007**, *64*, 938.
[93] N. K. Olrichs, M. E. G. Aarsman, C. J. Arnusch, B. de Kruijff, E. Breukink, T. den Blaauwen, unpublished results.
[94] M. Herve, A. Boniface, S. Gobec, D. Blanot, D. Mengin-Lecreulx, *J. Bacteriol.* **2007**, *189*, 3987.
[95] W. A. Weppner, F. C. Neuhaus, *J. Biol. Chem.* **1978**, *253*, 472.
[96] V. van Dam, R. Sijbrandi, M. Kol, E. Swiezewska, B. de Kruijff, E. Breukink, *Mol. Microbiol.* **2007**, *64*, 1105.
[97] M. A. Kol, A. I. de Kroon, D. T. Rijkers, J. A. Killian, B. de Kruijff, *Biochemistry* **2001**, *40*, 10500.
[98] V. Van Dam, B. De Kruijff, E. Breukink, **2008**, unpublished results.
[99] N. Nouwen, A. J. Driessen, *Mol. Microbiol.* **2002**, *44*, 1397.
[100] J. E. Moses, A. D. Moorhouse, *Chem. Soc. Rev.* **2007**, *36*, 1249.
[101] J. van Heijenoort, *Glycobiology* **2001**, *11*, 25R.

Received: October 10, 2008
Published online on January 28, 2009

