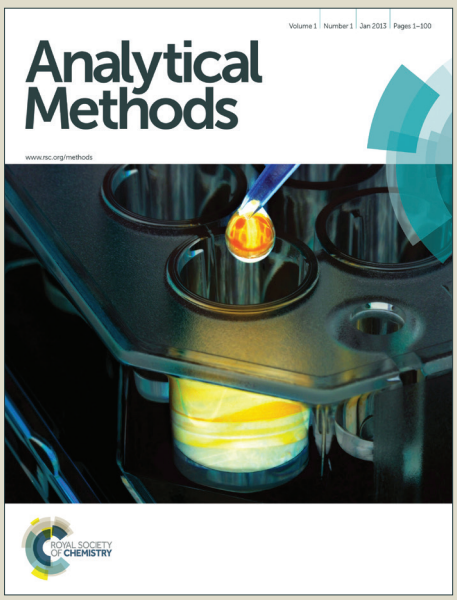
# Analytical Methods

Accepted Manuscript





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## Abstract

Transpeptidation as the last step in peptidoglycan synthesis has been a successful antibacterial target for many decades, whereas inhibitors of the preceding transglycosylation did not lead to any commercialized drugs although both reactions are essential for cell survival. Since the substrate, Lipid II, became more easily available, a lot of research towards synthesis of new inhibitors of transglycosylation has been done. Several assay types have been elaborated to evaluate them. The main problem in the development is the lack of UV-chromophore in the substrate causing the need for radioactive and fluorescent labeling. Also, high-throughput screens that allow screening of large libraries of compounds have been conceived. This review gives an overview of the different assay types and highlights some cleverly engineered screens.


# Overview of analytical methods for monitoring bacterial transglycosylation

Bart Blanchaert, Erwin Adams and Ann Van Schepdael

## 1. Introduction

The bacterial cell wall provides bacteria with a cell shape and protects them from osmotic pressure. There is a wide variety in shapes between several species, for instance spherical, helical, rod- or comma shaped. Although the main component, peptidoglycan, can vary significantly in structure and modifications [1], its integrity is crucial for all bacterial species. Therefore it has been an interesting therapeutic target for many years and the use of drugs targeting the cell wall, such as β-lactams and glycopeptides, is widespread. Increasing resistance to these antibiotics has become a major threat to human health, as already 60 % of the *S. aureus* isolates were found to be resistant to methicillin, oxacillin or nafcillin [2]. Several resistance mechanisms towards both β-lactams and glycopeptides have been described [3]. The antibiotics mentioned above were conceived as transpeptidase inhibitors whereas transglycosylase inhibitors have not yet found their way into human medicinal use. The aim of this review is to discuss the development of analytical methods to test and evaluate potential new antibacterial drugs targeting bacterial transglycosylation.

# 2. Peptidoglycan

## *2.1. Structure and characteristics*

The main component of the cell wall is peptidoglycan which consists of linear glycan strands cross-linked by short peptides. The glycan chains are composed of alternating N-acetylglucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues connected through β-1,4 bonds. Although glycan chain length distribution within one species is very broad, each species has a specific length for the majority of chains [1,4-6]. The sequence of the peptide, attached to the MurNAc by an amide bond [7], is L-Ala- γ-D-Glu- L-Lys- D-Ala- D-Ala in Gram-positive bacteria. In Gram-negative bacteria the L-lysine at position three is replaced by meso-diaminopimelic acid [4,8-9]. Several variations in the pentapeptide sequence as well as modifications to the glycan strands have been described in literature [1]. However a detailed overview is beyond the scope of this study.

## *2.2. The transglycosylation reaction*

Peptidoglycan is assembled from the last monomeric building block, Lipid II, in a two-step extracellular reaction. Lipid II consists of a head group (N- acetylmuramoyl- (N-acetylglucosamine)- pentapeptide) coupled to a lipid undecaprenol carrier by a pyrophosphate [10]. In a first step, the C4 of GlcNAc of lipid II is coupled to the C1 of MurNAc in the nascent glycan strand by a glycosidic bond and thereby releasing the undecaprenyl diphosphate carrier [4,11]. The glycan chain is extended by addition of new units to the reducing end (where undecaprenyl pyrophosphate is coupled)[12]. This reaction is called transglycosylation and causes the glycan chains to grow in length. Subsequently transpeptidation occurs in which the peptides are cross-linked. The most common form of cross linking occurs between the amino acid in the third position and the carboxyl group of alanine at position 4 of another glycan strand, either directly (Gram-negative species) or via an interpeptide bridge (most Gram-positive species) [1,13]. These reactions are shown in Figure 1.

Both reactions are catalyzed by a class of membrane-bound enzymes called ‘Penicillin Binding Proteins’ (PBPs) [9]. The PBPs are commonly divided into two classes; class A and B. While Class B enzymes can only catalyze transpeptidation, Class A enzymes have an N-terminal transglycosylation site as well, making them capable of catalyzing both

reactions [4,14,15]. Besides PBPs, monofunctional glycosyl transferases (MGT), enzymes with non PBP-related transglycosylation activity, have been discovered in several Gram-positive and -negative organisms [16]. However, because of their high degree of similarity, they are likely to be sensitive to the same inhibitors [16,17].

The undecaprenyl pyrophosphate tail is released upon transglycosylation and subsequently flipped towards the cytosolic side of the plasma membrane where it is dephosphorylated to the monophosphate and can be used to regenerate Lipid II [10]. It is assumed that the flexible undecaprenyl chain interacts in the flipping process although the precise mechanism and the driving force for it have not been elucidated [18-20]. The biosynthesis of new Lipid II molecules involves multiple enzymatic reactions and could be an attractive drug target as well [21]. Since Lipid II is not abundantly present in the cell membrane, this process is believed to have a very high turnover [10].

# 3. Development of assays for bacterial transglycosylation inhibitors

Transglycosylation has been intensively studied since the substrate became available in larger quantities due to efforts in chemical synthesis [22-24] and the development of a membrane-bound enzymatic pathway for Lipid II synthesis [25]. The lack of a UV-chromophore in Lipid II urges the development of analysis methods based upon different detection modes.

## *3.1. Radioactive labeling*

### 3.1.1. Chromatography based analysis

In the oldest transglycosylation assay types, a radiolabel is incorporated in Lipid II. The transglycosylation rate is determined by paper chromatography analysis in which the produced glycan chains have a very low retention factor and are thus immobilized near the loading spot. Quantitation is performed by a scintillation counter or a phosphor imager scanner [26-28]. The paper chromatography is conserved throughout the years as isobutyric acid-l M ammonia (5:3) is still the most commonly used mobile phase. The [$^{14}$C]-radioactive label can be incorporated in various moieties of Lipid II. In most recent assays meso-diaminopimelic acid or N-acetylglucosamine are the label-bearing residues [17,28-32], although lysine, alanine, and glycine have been used as well [33]. Also the

use of [$^3$H] labeled meso-diaminopimelic acid has been described [34]. Radiolabels can be incorporated either by acetylation or by enzymatic production of Lipid II using radiolabeled precursors [35]. Paper chromatography causes these assays to be very time-consuming (the drying step alone takes about 6 hours) and prohibits high-throughput screening for antibiotics [28]. Obtaining results using these assays is also not straightforward and requires many manipulations of radiolabeled substances. Nevertheless, this method is still in use to characterize the active site of various transglycosylases such as *E.coli* PBP1b [29], *S. aureus* MGT [17] and PBP2 [30]. Also the effect of modification of Lipid II and isolation of the active site on transglycosylation have been investigated [28,32,33,36-38]. These modifications are aimed at increasing water solubility which is more convenient for assay development. In an assay using *E. coli* membranes instead of isolated recombinantly produced enzymes, Lipid II analogues with shorter isoprenyl chains were found to be suitable substrates for transglycosylation [33]. Lipid IV, an analogue containing 2 disaccharide moieties, seemed to have higher affinity for *E.coli* PBP1b than Lipid II [24]. Paper chromatography and even TLC analysis are being used by groups that test chemically modified analogues of Lipid II or known antibiotics for inhibitory effects on transglycosylation [39-41].

Barrett et al. [35] replaced paper chromatography by SDS-PAGE analysis (Fig. 2), which significantly reduced analysis time and allowed investigation of variance in glycan chain length distributions as a result of modifications to enzyme or substrate. SDS-PAGE can separate strands up to 10 disaccharide units, larger strands merge as a smear [42]. Recently, an HPLC assay based upon radioactive detection has been developed by Biboy et al. [43]. Prior to HPLC analysis, glycan strands are digested with muramidase and reduced with sodium borohydride to yield muropeptides, the disaccharide peptide units of peptidoglycan. Reduction is done to improve muropeptide separation as the 2 anomeric forms of MurNAc are converted into muramitol. Muropeptides are detected using a flow-through radioactivity detector [43].

### 3.1.2. Scintillation proximity assay

A scintillation proximity assay (SPA) has been developed for peptidoglycan synthesis. For these assays beads coated with wheat germ agglutinin which specifically binds cross-linked peptidoglycan are used [44]. Inside the beads is a scintillant that emits light after

stimulation which occurs when radio-labeled compounds attach to the surface [45]. This setup offers more high-throughput capabilities compared to the time-consuming paper chromatography analysis. Substrate for these assays is UDP-[$^3$H]GlcNAc which is a precursor for Lipid II and is more readily available than radioactively labeled Lipid II. Instead of isolated transglycosylases, membrane fragments are extracted from bacteria. In this manner, enzymes involved in production of Lipid II such as MraY, which couples UDP-MurNAc-pentapeptide to the undecaprenyl phosphate, and MurG, catalyzing the addition of UDP-[$^3$H]GlcNAc to form Lipid II, are monitored simultaneously with transglycosylases and transpeptidases in one assay [21]. Subsequent to quenching the reaction with EDTA, the wheat germ agglutinin coated SPA beads are added and cross-linking can be measured [44,46,47]. Drawback of these assays is that activity of an antimicrobial compound cannot be attributed to a specific enzyme and kinetic parameters cannot be determined [48,49]. Compounds interfering with cell viability or with the production of peptidoglycan through an unrelated mechanism can results in false positives. Also, the fact that wheat germ agglutinin is not a specific antibody, can increase the ratio of false positives. Despite the relatively low incidence of artifacts in SPA especially in radiochemical assays, false positive results are always possible. Like for all screening methods, hits have to be confirmed in orthogonal assays [45].

### *3.2. Fluorescent labeling*

General drawbacks of working with radioactive labels are commonly known. To overcome these problems, several groups have made successful attempts to replace radioactive labels with fluorescent tags. Pre incubation coupling of a dansyl group to the primary amine of lysine in Lipid II is most commonly used [42,50-52], but members of the Alexa fluor family can be suitable too [52]. Post incubation labeling with fluorescamine onto the primary amine has been proposed as well [22,50]. Although post incubation tagging is more time-consuming since an extra reaction step is required each time, it precludes any influence of the label on the kinetic parameters or on the affinity of inhibitors for the peptide moiety of Lipid II. One could imagine a possible influence of a bulky fluorescent group attached to lysine on the affinity of vancomycin for its target, D-Ala-D-Ala, which is situated next to lysine [54]. Any label on the lysine residue renders transpeptidation impossible because of the importance of lysine for this reaction.

However, a mixture of 95 % unlabeled Lipid II and 5 % of the dansylated variant allows simultaneous transglycosylation transpeptidation assays as only a minority of peptide residues in peptidoglycan is cross-linked [42].

Like many of the assays depending on radiolabels described above, fluorescence based assays have also been used to characterize the transglycosylation site and to study the influence of modifications to enzyme or substrate. Helassa et al. developed an assay based upon SDS-PAGE with dansylated Lipid II. They observed that native *S. pneumoniae* PBP2a has similar kinetic parameters for transglycosylation compared to a truncated form consisting only of the periplasmic part [42]. However, since inhibitors such as moenomycin only recognize the transglycosylation site when the transmembrane segment of PBPs is present, it is better to use native enzymes in antibacterial drug screens [52]. Also, a much simpler SDS-PAGE test that can only evaluate affinity of dansylated Lipid II for truncated *S. pneumoniae* PBP2a* has been developed. In the same study, TLC was used to monitor the *in vitro* transglycosylation reaction [51].

Fluorescent labels offer the possibility of HPLC with a fluorescence detector, which is likely to obtain a better sensitivity and precision than aforementioned techniques. HPLC offers multiple interesting separation mechanisms for this application such as reversed phase [55], anion exchange [22,50,55] or size exclusion chromatography [53] (Fig. 3). Because HPLC analysis usually takes less than one hour and can easily be automated, it seems to be the method of choice in recent studies. Liu et al. developed an interesting variant. They presumed that an incorporation of the label into the undecaprenyl tail of Lipid II is not expected to change the affinity towards inhibitors. Since the lipid anchor is shielded from antibacterial agents by the cell membrane *in vivo*, it is not a possible target for antibacterial drugs anyway [55]. Also, the fact that the label will not be incorporated in the peptidoglycan prevents its interference in the chromatogram. Furthermore, since the label is in the lipid tail, which is detached during transglycosylation, attachment of a Förster resonance energy transfer (FRET) donor to the peptide moiety offers possibilities of high-throughput.

# 4. High-throughput screening efforts

In the search for new drug leads, high-throughput screening is the best tool to test a library of compounds. Found hits should then be confirmed in the assays described above and their inhibition constants can be determined.

## *4.1. Transglycosylase affinity screens*

The simplest type of high-throughput screens is based upon a competition of a potential drug lead and moenomycin A for binding the transglycosylation site. The detection principle can vary between different assays. Vollmer et al. immobilized moenomycin A on activated agarose and labeled PBPs in membrane extracts using [$^3$H]benzylpenicillin. Beads were added to 96 well plates containing moenomycin A, membrane extracts and the test substance. A decrease in radioactivity after a washing step meant the test compound had affinity for the transglycosylation site and could compete with moenomycin A. Moenomycin analogues with lower affinity could also be used for identification of less potent inhibitors [56,57]. A similar screen can be conceived using fluorescence anisotropy. It was observed that the anisotropy of a fluorescently labeled moenomycin increased upon addition of PBPs and the increase was lost upon addition of competitive binders to the transglycosylation site [52,58]. A hit was identified following a 90 % reduction in anisotropy. Screens like these allow a relatively simple high-throughput evaluation of large libraries. However, their simplicity is also their main weakness as only transglycosylation inhibitors directly targeting PBPs can be detected. So far, only the moenomycin family and some derivatives are known to block transglycosylation in this manner [59]. A similar screen has been developed using surface plasmon resonance, but since this is not amenable for high-throughput it is not commonly used [60].

## *4.2 Transglycosylase activity screens*

Efforts to analyze the transglycosylation reaction in a high-throughput format have been most valuable. Already in 2002 Schwartz et al. developed their so called continuous fluorescence assay [50]. It was based upon the observation that the quantum yield of the dansyl fluorophore is dependent on the surroundings, being higher in a more hydrophobic environment. Dansylated Lipid II was solubilized by addition of decyl PEG and was

incubated in a buffer supplemented with *E. coli* PBP1b and muramidase, which cleaved the growing chains to yield the dansylated water soluble disaccharide-pentapeptide monomer. The transfer of the fluorophore out of the micelles caused a measurable decrease in fluorescence. A schematic overview is given in Figure 4. A drawback is that a measurement of such a small decrease in fluorescence might not be robust enough for high-throughput screening. The continuous fluorescence assay was validated by monitoring the same reactions using anion exchange HPLC. Offant et al. adapted this principle to a 96 well format for the study of *T. maritime* PBP1a [61]. This assay has proven its value in high-throughput screening [62]. An additional advantage is the opportunity for continuous monitoring which revealed a lag phase [50]. The authors postulated this to be due to a more efficient elongation of the chain when a primer strand is present. This was later confirmed and a probe bypassing the lag phase was developed. A galactose residue blocks the probe at the reducing end to ensure that it can only serve as a primer for a new strand [63].

As mentioned before [55], adding a FRET-donor is an interesting strategy. Researchers from the same group developed a FRET-Based Lipid II Analogue (FBLA) which contained a coumarin fluorophore attached to lysine and a quencher coupled to 5 instead of 11 isoprene units (Fig. 5) [64]. Due to the FRET principle, this quencher caused intact FBLA to not have any fluorescence at all, in contrast to the screen developed by Schwartz. After transglycosylation and subsequent cleavage by muramidase, the distance between quencher and fluorophore will increase immensely and fluorescence can be observed. This screen has been performed in a 1536 well format and has been validated with several known inhibitors as positive control and by analysis with HPLC. An excess of muramidase is added to immediately cleave all nascent strands and avoid the lag phase as described above.

## 5. Conclusion

The importance of the bacterial cell wall as a therapeutic target has been understood for decades and led to the success of drugs such as the β-lactam antibiotics and glycopeptides. However, as they were discovered as transpeptidase inhibitors, the first drug to be conceived as transglycosylase inhibitor has yet to reach the market. An overview was given of several attempts aimed at both characterization of enzymes with a

transglycosylation site and the search for new inhibitors. Old transglycosylation assays that can still prove their value in characterization of the active site and evaluation of modifications to the substrate have been described. These assays have been an inspiration for the development of some excellent new high-throughput screens in the last few years. They will hopefully help researchers to find drug leads that can eventually reach the market of human therapeutic use and that can, just like penicillin more than 80 years ago, be the start of a new success story in antibacterial therapy.

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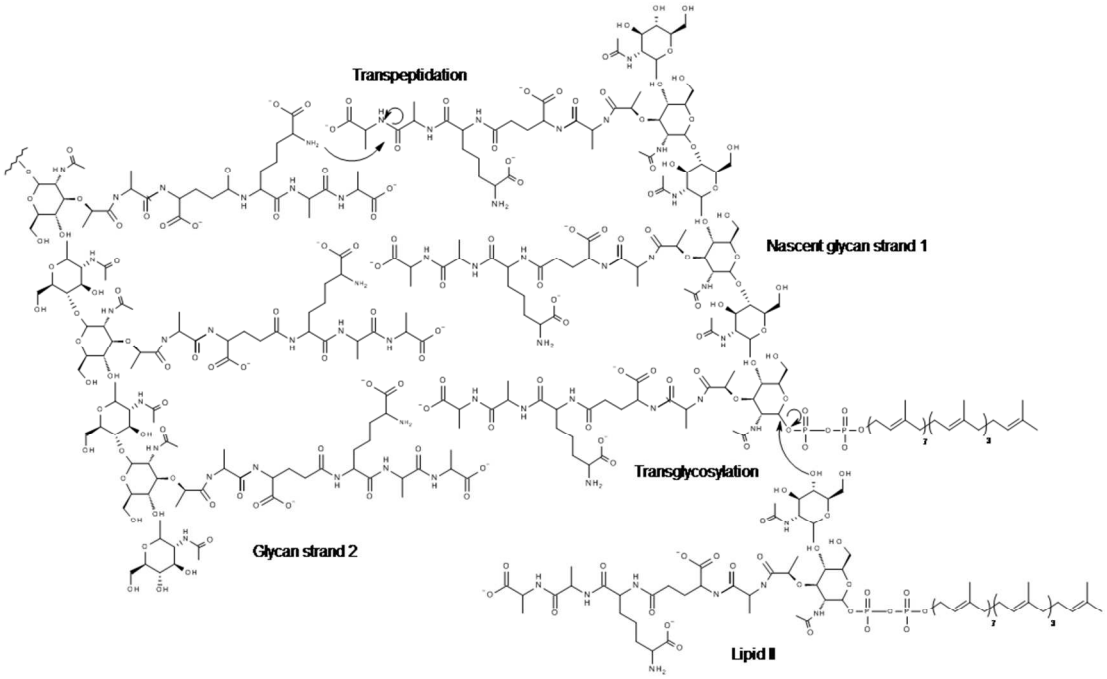


Figure 1: Scheme of transglycosylation and transpeptidation. Glycan strands are extended by addition of Lipid II units to the reducing end and a release of the undecaprenyl pyrophosphate tail. The Lipid II depicted here is the Gram-negative form containing meso-diaminopimelic acid instead of the lysine residue present in the Gram-positive form. Additional mechanistic information on transpeptidation can be found in [65].

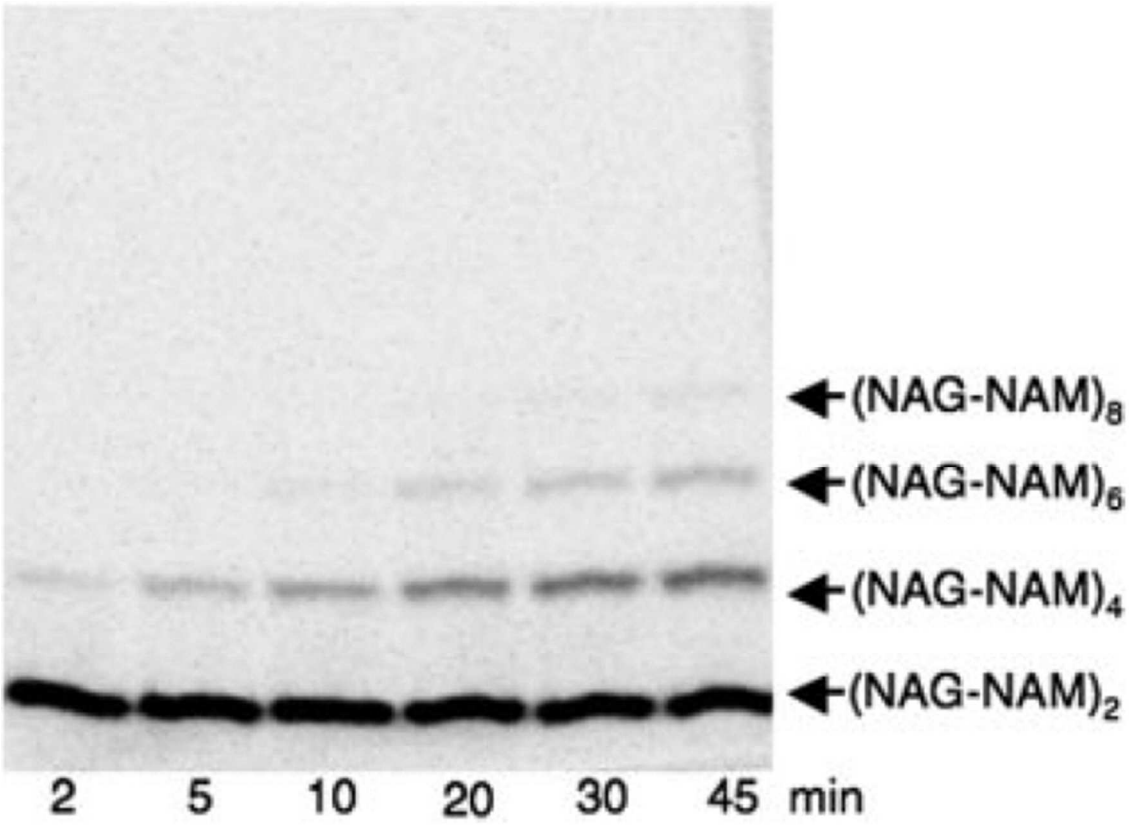


Figure 2: SDS-PAGE analysis of transglycosylation products of E. coli PBP1A. Aliquots were taken and inactivated over various time points. Conjugated oligomers were separated according to size. Chain length increased with 2 disaccharide units because Lipid IV was used as substrate. Picture adapted from [34].

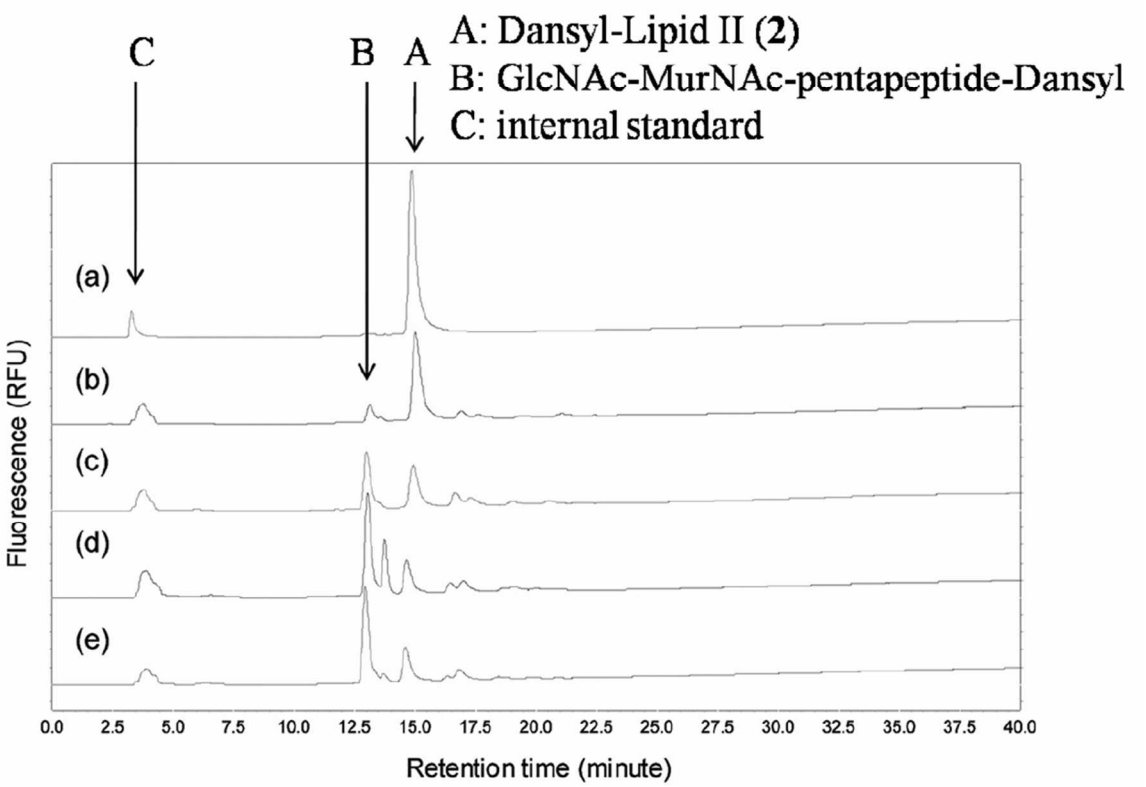


Figure 3: Anion exchange HPLC-FD chromatogram obtained after analysis of a transglycosylation reaction. Dansylated Lipid II was incubated with C. difficile PBP and growing strands were cleaved by muramidase. The incorporation of the fluorescent tag in the disaccharide pentapeptide group allows simultaneous monitoring of the substrate and the reaction product. Traces a–e are for the starting material (t = 0) and the reaction mixtures at, t = 1, 2, 3, and 4 h, respectively. Adapted with permission from C. Y. Liu, C. W. Guo, Y. F. Chang, J. T. Wang, H. W. Shih, Y. F. Hsu, C. W. Chen, S. K. Chen, Y. C. Wang, T. J. Cheng, C. Ma, C. H. Wong, J. M. Fang and W. C. Cheng, Org Lett., 2010, 12, 1608-1611. Copyright 2010 American Chemical Society.

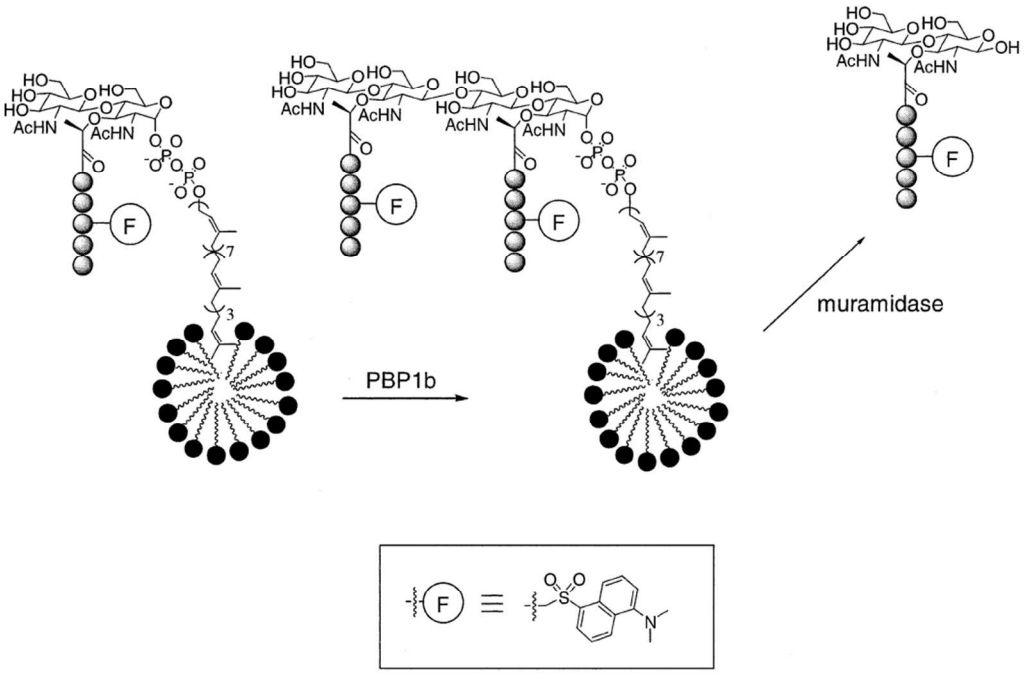


Figure 4: Scheme of the continuous fluorescence assay. Dansylated Lipid II trapped inside the micelles is built into a nascent glycan strand by E. coli PBP1b. The disaccharide-pentapeptide will subsequently be cleaved off by muramidase allowing it to transfer to a more aqueous environment where fluorescence will decrease. Reprinted with permission from B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang and R. L. Stein, Biochemistry, 2002, 41, 12552-12561. Copyright 2002 American Chemical Society

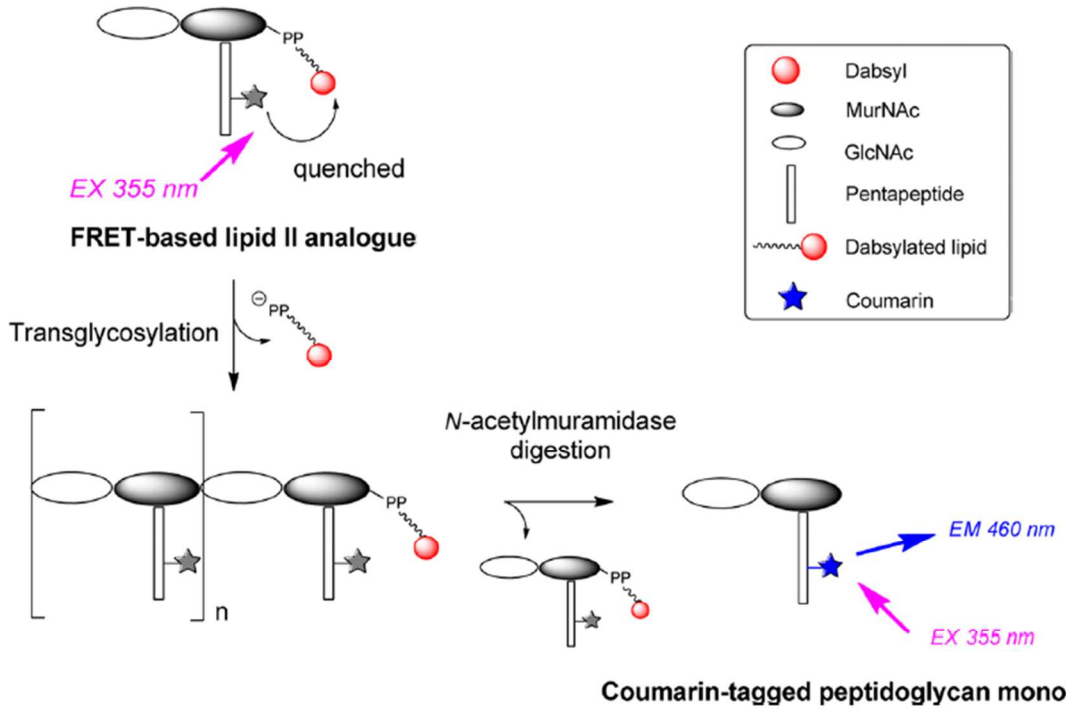


Figure 5: The FRET quencher in the FRET based Lipid II analogue (FBLA) precludes fluorescent detection. During transglycosylation, the dabsylated lipid tail is released from FBLA. After subsequent digestion by muramidase the distance between the FRET donor and acceptor increases so vastly, the FRET quenching is lost and the coumarin tagged monomer can be detected. Adapted with permission from S. H. Huang, W. S. Wu, L. Y. Huang, W. F. Huang, W. C. Fu, P. T. Chen, J. M. Fang, W. C. Cheng, T. J. Cheng, C. H. Wong, J Am Chem Soc., 2013, 135, 17078-17089. Copyright 2013 American Chemical Society.