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## Small molecule inhibitors of peptidoglycan synthesis targeting the lipid II precursor

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1 **Small molecule inhibitors of peptidoglycan synthesis targeting the lipid II precursor**

2 **Running title: Small molecules targeting lipid II**

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7 4 Adeline Derouaux<sup>a</sup>, Samo Turk<sup>b</sup>, Nick K. Olich<sup>c</sup>, Stanislav Gobec<sup>b</sup>, Eefjan Breukink<sup>c</sup>, Ana  
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9 5 Amoroso<sup>a</sup>, Julien Offant<sup>d</sup>, Julieanne Bostock<sup>e</sup>, Katherine Mariner<sup>e</sup>, Ian Chopra<sup>e</sup>, Thierry  
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11 6 Vernet<sup>d</sup>, Astrid Zervosen<sup>f</sup>, Bernard Joris<sup>a</sup>, Jean-Marie Frère<sup>a</sup>, Martine Nguyen-Distèche<sup>a</sup>, and  
12  
13  
14 7 Mohammed Terrak<sup>a,\*</sup>  
15  
16  
17 8

18  
19 9 <sup>a</sup> Centre d'Ingénierie des Protéines, Université de Liège, Allée de la chimie, B6a, B-4000, Sart  
20  
21  
22 10 Tilman, Liège, Belgium.

23  
24 11 <sup>b</sup> University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia.

25  
26 12 <sup>c</sup> Department Biochemistry of Membranes, Bijvoet Center, Institute of Biomembranes,  
27  
28  
29 13 Utrecht University, Utrecht 3584 CH, The Netherlands  
30

31 14 <sup>d</sup> Institut de Biologie Structurale (UMR 5075, Université Joseph Fourier, CNRS, CEA), Grenoble,  
32  
33  
34 15 France

35  
36 16 <sup>e</sup> Institute of Molecular and Cellular Biology and Antimicrobial Research Centre, University  
37  
38  
39 17 of Leeds, Leeds LS2 9JT, U.K.

40  
41 18 <sup>f</sup> Centre de Recherches du Cyclotron, Allée du 6 Août, 8, B30, Université de Liège, 4000  
42  
43  
44 19 Liège, Belgium.

45  
46 20  
47  
48 21 **\*Corresponding author:** Mohammed Terrak, Centre d'Ingénierie des Protéines, Université  
49  
50  
51 22 de Liège, Allée de la Chimie, B6a, B-4000, Sart Tilman, Liège, Belgium.

52  
53 23 Tel.: 32-4-3663332; Fax: 32-4-3663364; E-mail: [mterrak@ulg.ac.be](mailto:mterrak@ulg.ac.be).  
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## 1 Abstract

2 Bacterial peptidoglycan glycosyltransferases (GTs) of family 51 catalyze the polymerization  
3 of the lipid II precursor into linear peptidoglycan strands. This activity is essential to bacteria  
4 and represents a validated target for the development of new antibacterials. Application of  
5 structure-based virtual screening to the National Cancer Institute library using eHits program  
6 and the structure of the glycosyltransferase domain of the *Staphylococcus aureus* penicillin-  
7 binding protein 2 resulted in the identification of two small molecules analogues 5, a 2-[1-[(2-  
8 chlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine and 5b, a 2-[1-[(3,4-  
9 dichlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine that exhibit  
10 antibacterial activity against several Gram-positive bacteria but were less active on Gram-  
11 negative bacteria. The two compounds inhibit the activity of five GTs in the micromolar  
12 range. Investigation of the mechanism of action shows that the compounds specifically target  
13 peptidoglycan synthesis. Unexpectedly, despite the fact that the compounds were predicted to  
14 bind to the GT active site, compound 5b was found to interact with the lipid II substrate via  
15 the pyrophosphate motif. In addition, this compound showed a negatively charged  
16 phospholipid-dependent membrane depolarization and disruption activity. These small  
17 molecules are promising leads for the development of more active and specific compounds to  
18 target the essential GT step in cell wall synthesis.

19  
20  
21 **Keywords:** Glycosyltransferase; Peptidoglycan; Antibacterial; Lipid II; Penicillin-Binding  
22 Protein.

## 1 1. Introduction

2  
3  
4  
5 3 The increase of bacterial resistance to antibiotics has resulted in a decline of available  
6  
7 4 efficient antibacterial treatments. Therefore, the discovery and development of new antibiotic  
8  
9 5 classes able to cure infections due to resistant pathogens are urgently needed.

10  
11 6 Peptidoglycan is an essential polymer and the main constituent of the bacterial cell  
12  
13  
14 7 wall. Its biosynthesis requires several steps and offers many unexplored targets for the  
15  
16  
17 8 development of new antibacterial drugs [1, 2]. The last two reactions in peptidoglycan  
18  
19 9 biosynthesis are performed by bifunctional penicillin-binding proteins (PBPs) and result in the  
20  
21  
22 10 assembly of the cell wall polymer from the monomeric intermediates [3]. This takes place  
23  
24 11 outside the plasma membrane and relies on the activity of the glycosyltransferase (GT)  
25  
26  
27 12 module of bifunctional PBPs which uses the lipid II precursor to synthesize glycan chains and  
28  
29 13 their transpeptidase module which catalyzes the cross-linking of two glycan chains via the  
30  
31  
32 14 peptide side chains. Inhibition of either of these two reactions leads to bacterial cell death.  $\beta$ -  
33  
34 15 Lactam antibiotics target the transpeptidation reaction but antibiotic therapy based on  
35  
36  
37 16 inhibition of the GTs has not yet been developed. The only well characterized inhibitor  
38  
39 17 targeting the GTs is the natural product moenomycin, a potent antibacterial phosphoglycolipid  
40  
41  
42 18 active at nanomolar concentrations [4]. Despite intensive studies of its structure-function  
43  
44 19 properties it has not been developed for use in human chemotherapy because of poor  
45  
46  
47 20 pharmacokinetic properties related to its C25 lipid chain. A delipidated moenomycin is  
48  
49 21 inactive and the sugar moiety can only be reduced to three saccharide units while retaining  
50  
51  
52 22 good antibacterial activity [4].

53 23 Recently the X-ray structures of PBPs or GT modules and their complexes with  
54  
55  
56 24 moenomycin A have been determined [5-8]. They shed light on the GT domain fold, which  
57  
58  
59 25 exhibits some similarities with that of  $\lambda$ -lysozyme and confirmed the catalytic mechanism of

1 glycan chains elongation and the mode of action of moenomycin. The GT domain contains an  
2 extended enzymatic cleft which can accommodate six sugar units. It is divided into two sub-  
3 sites, a donor site for the elongating chain and an acceptor site for the lipid II substrate.  
4 Between the sub-sites a flexible region is proposed to play an important role in the  
5 translocation of the product from the acceptor site to the donor site through a folding and  
6 unfolding process [9]. The enzymatic cavity is bordered by several conserved residues and  
7 harbors the two glutamate residues essential for catalysis [10].

8 Inhibition of the GT reaction can be accomplished either by a compound binding to  
9 the enzyme, like moenomycin which occupies the donor site mimicking the elongating glycan  
10 chain [5], or by agent binding to the lipid II substrate [11]. Natural products such as nisin are  
11 known to target the peptidoglycan precursor. Nisin binds to the pyrophosphate motif of lipid  
12 II, sequesters the substrate and induces pore formation in the bacterial membrane.  
13 The potential of membrane damaging agents as antibacterials has been validated in the case of  
14 the cyclic lipopeptide daptomycin, a drug used in the treatment of certain infections caused by  
15 Gram-positive bacteria. Its antibacterial activity against *Staphylococcus aureus* including  
16 methicillin-resistant strains has been shown to be the result of membrane potential disruption  
17 [12].

18 In this paper we report the discovery of new peptidoglycan GT inhibitors through the  
19 use of structure-based virtual screening of small molecules from the National Cancer Institute  
20 library. Selected hits were evaluated for their ability to inhibit in vitro the GTs activity of  
21 *Escherichia coli* PBP1b in the presence of lipid II substrate, followed by the determination of  
22 their effect on bacterial growth. The active compounds were then submitted to several assays  
23 to demonstrate their specificities and modes of action.

## 1 2. MATERIALS AND METHODS

### 3 2.1. Chemicals

4 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-  
5 (1-glycerol)] (DOPG), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(3-lysyl(1-glycerol))] (lysyl-  
6 DOPG) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from  
7 Avanti Polar Lipids Inc. Nisin A was produced, isolated, and purified as described [13].  
8 Moenomycin A, was a gift from Aventis (France). The fluorescent dye 3,3'-  
9 diethylthiodicarbocyanine iodide (DiSC<sub>2</sub>(5)) was from Molecular Probes Inc. Lipid I, Lipid II  
10 and Dansyl-lipid II were synthesized and purified as described elsewhere [14].  
11 Undecaprenylphosphate and undecaprenylpyrophosphate were obtained by phosphorylation  
12 of undecaprenol [15] that was isolated from *Laurus nobilis* as described [16]. Radiolabeled  
13 [<sup>14</sup>C]*meso*-diaminopimelic acid (A<sub>2</sub>pm)-labelled lipid II was prepared essentially as  
14 previously described [17].  
15 The small molecules tested in this study were obtained from the National Cancer Institute and  
16 solubilised in DMSO at a concentration of 100 mM or 10 mM depending on the solubility of  
17 the compound. The identities of the active compounds 5 and 5b (NSC n° 17383, and NSC n°  
18 17382 respectively) were verified by mass spectrometry and NMR. The data (not shown)  
19 were consistent with the expected mass and structure for both compounds.

### 21 2.2. Proteins expression and purification

22 Five model proteins of the GT51 family were prepared and used in this study. *E. coli* PBP1b,  
23 *S. aureus* MtgA and PBP2, and the PBP1a from *Thermotoga maritima* MSB8 were produced  
24 and purified as previously described [5, 17-19]. The gene encoding (A68-Q723) PBP2 of  
25 *Enterococcus hirae* was cloned into pET28a (+) expression plasmid (Novagen) and the His

1 Tag PBP2 was expressed in *E. coli* BL21 (DE3). The cells were grown at 37°C to an optical  
2 density of 0.8 at 600 nm, protein expression was induced with 0.5 mM isopropyl  $\beta$ -D-1-  
3 thiogalactopyranoside and incubation was continued overnight at 18°C. The cells were  
4 resuspended in 25 mM HEPES pH 7.5, 500 mM NaCl and the PBP2 was purified in one step  
5 on a HisTrap column (GE, Heathcare) (unpublished results). The total protein yield was about  
6 15 mg per litre of culture and the purity was over 90% as judged by on SDS-PAGE.

### 7 8 **2.3. Virtual screening**

9 The virtual screening was performed using the programme eHiTS 6.0 from SimBioSys Inc.  
10 eHiTS (<http://www.simbiosys.com/ehits>) [20, 21] was used for the active site detection and  
11 docking. Open Babel (<http://openbabel.org>) was used for manipulating the ligands with  
12 various chemical formats. PyMol from DeLano Scientific was used for visual inspection of  
13 the results and the graphical representations.  
14 The 3D structures of the compounds from the NCI Diversity Set were obtained from the NCI  
15 webpage (<http://dtp.nci.nih.gov/dw/testmasters/chem3d.html>). The 1990 highly diverse  
16 compounds of the NCI Diversity Set represented a broad chemical spectrum of the whole NCI  
17 database. However, no special preparation of the 3D structures was applied since eHiTS  
18 automatically evaluates all the possible protonation states for the ligands and enzymes. The  
19 crystal structure of *S. aureus* PBP2 as a complex with moenomycin (PDB entry 2OLV) [5]  
20 was initially prepared with eHiTS. The program automatically detected the ligand in the  
21 complex and selected the part of the enzyme within a 7Å margin around the ligand as the  
22 active site. The NCI Diversity Set was then docked into the active site. The scoring was  
23 according to the eHiTS\_Score that is included in the eHiTS software package. Two-  
24 dimensional similarities search was performed using a ZINC built-in engine and the entire  
25 NCI database containing more than 250 000 compounds (Tanimoto similarity index was set to



1 0.75).

2

## 3 **2.4. In vitro glycosyltransferase activity and GT inhibition assays**

### 4 **2.4.1. Fluorescence assay.**

5 GT activity was monitored using the continuous fluorescence assay method [22]. For the  
6 simultaneous screening of numerous conditions this technique was adapted to a 96-well plate  
7 format (Greiner Bio-One). The standard reaction was carried out at 30°C in 50 µl of 50 mM  
8 Hepes pH 7.5, 200 mM NaCl, 0.2% decyl PEG, 10 mM CaCl<sub>2</sub>, 20% DMSO, 10 µM dansyl-  
9 lipid II, 1 unit of muramidase (Cellosyl) and 100 nM *E. coli* PBP1b. The data were collected  
10 for 30 min using a Victor 3 fluorimeter (Perkin Elmer) with excitation at 355 nm and  
11 emission at 536 nm.

12 For the other GT enzymes the reaction conditions were adapted for optimal activity as  
13 follows, with only variable conditions given: 2 µM *S. aureus* MtgA was used in the presence  
14 of 20 µM dansyl-lipid II, 10% DMSO and 10 mM MnCl<sub>2</sub>. 2.5 µM *S. aureus* PBP2 was used  
15 in the presence of 20 µM dansyl-lipid II and 50 mM sodium acetate pH 5. *E. hirae* PBP2 and  
16 *T. maritima* PBP1a were used at 300 nM and 150 nM respectively.

### 17 **2.4.2. Radioactive assay.**

18 The radioactive assay was performed in 30 µl in the same conditions as the fluorescence assay  
19 using 4 µM [<sup>14</sup>C]lipid II (0.126 µCi nmol<sup>-1</sup>) instead of the fluorescent substrate and by  
20 omitting the muramidase. The reaction was stopped with moenomycin (10 µM) and the  
21 products were separated by TLC in 2-propanol-ammonium hydroxide-H<sub>2</sub>O (6:3:1; V/V/V)  
22 and analyzed using a Typhoon Trio+ Imager and the ImageQuant TL software (GE  
23 Healthcare).

### 24 **2.4.3. GT inhibition assay.**

25 The IC<sub>50</sub> values of compounds 5 and 5b were determined using a fluorescence based assay.

1 The initial rate of the reaction was determined in the presence of various concentrations of  
2 inhibitor (20-1500  $\mu\text{M}$ ) and plotted versus the inhibitor concentration using the Sigma plot  
3 program (Systas Software). The  $\text{IC}_{50}$  value is the inhibitor concentration that decreases the  
4 initial rate by a factor of 2.

## 6 **2.5. MIC determination and effect of 5b on bacterial viability**

7 The *S. aureus*, *Enterococcus faecalis*, *Listeria innocua*, *Micrococcus luteus*, *Streptococcus*  
8 *epidermis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* strains listed in table 2  
9 were obtained from the American Type Culture Collection (ATCC). The *E. coli* strains 1411  
10 and SM 1411 were reported previously [23].

11 Minimum inhibitory concentration (MIC) determinations were carried out using the Clinical  
12 and Laboratory Standard Institute (CLSI, 2009) broth microdilution method. Compounds  
13 were solubilised in 100% DMSO at a concentration of 10 mg/ml, and twenty-fold diluted in  
14 Mueller-Hinton broth (MHB), just before utilization.

15 For bacterial viability experiments *S. aureus* ATCC 25923 colonies were grown  
16 overnight on Mueller-Hinton agar (MHA) plates and suspended in MHB ( $\cong 1.10^8$  CFU/mL).  
17 Compound 5b was added at 1, 2 and 4 times the MIC and mixed. Samples were taken at  
18 different time intervals of incubation at 37° C, serially diluted and plated on MHA in  
19 triplicate. The colonies were counted after incubation at 37° C for 18h.

## 21 **2.6. Pathway reporter assays in *Bacillus subtilis***

22 We have employed a *Bacillus subtilis* reporter system which differentiates inhibitors of cell  
23 envelope, DNA, RNA, protein and fatty acid biosynthesis by quantifying the upregulation of a  
24 specific luciferase reporter construct in each case as described [24, 25].

25 The assay based on the LiaRS two-component system in *Bacillus subtilis* that senses

1 stress on the cell wall caused by compounds interfering with lipid II cycle was used according  
2 to published methods [26, 27]. The *B. subtilis* strain BFS2470 carries a  $\beta$ -galactosidase  
3 reporter gene under the control of *lial* promoter. The effect of antibiotics or compounds  
4 spotted on a Whatman disc is revealed by blue/white selection method after overnight  
5 incubation at 37°C on agar-plate containing 5-bromo-4-chloro-3-indolyl-beta-D-  
6 galactopyranoside (X-Gal).

### 7 8 **2.7. LIVE/DEAD bacterial viability assay**

9 The membrane damage inflicted by 10 minutes exposure to a panel of agents on *S. aureus*  
10 SH1000 was analysed using the LIVE/DEAD *Baclight*<sup>TM</sup> bacterial viability kit as reported  
11 [46]. Compound 5b and several control antimicrobial agents were tested at 4x MIC in  
12 comparison to a drug free control.

### 13 14 **2.8. Preparation of large unilamellar vesicles (LUVs)**

15 Large unilamellar vesicles were prepared essentially as described [28]. Desired amounts of  
16 lipid solutions in chloroform were mixed and evaporated under a gentle stream of nitrogen.  
17 The lipid film was subsequently dried for 20 min under vacuum. The film was hydrated by the  
18 addition of the buffer of choice under mechanical agitation and submitted to ten freeze-thaw  
19 cycles using liquid nitrogen and a water bath. The lipid suspension was then extruded ten  
20 times through a polycarbonate membrane filter with a pore size of 200 nm (Whatman  
21 International) [29]. The final phospholipid concentration was determined by phosphate  
22 analysis according to Rouse *et al.* [30].

### 23 24 **2.9. Vesicles binding assay**

25 DOPC with or without 1 mol% Lipid II vesicles were prepared as described above in 10 mM

1 MES-KOH, 15 mM K<sub>2</sub>SO<sub>4</sub> at pH 7. Vesicles (1 mM lipid Pi) were incubated with 5 μM and  
2 20 μM 5b, respectively, for 15 min at room temperature. The mixture was centrifuged in a  
3 TLA 120.2 rotor using a Beckman Ultracentrifuge (TL-100) for 1.5 h at 100 krpm and 20 °C.  
4 The amount of 5b before centrifugation and in the supernatant and pellet was determined by  
5 monitoring its intrinsic fluorescence on a Cary Eclipse fluorescence spectrophotometer  
6 (Varian Inc.). The percentage of 5b in the supernatant and pellet was determined by  
7 comparing the maximal value (350 nm) of fluorescence emission intensity.

### 9 **2.10. Carboxyfluorescein leakage assay in large unilamellar vesicles**

10 Carboxyfluorescein (CF)-loaded LUVs were prepared in 50 mM MES-KOH, 100 mM K<sub>2</sub>SO<sub>4</sub>,  
11 pH 6.5 (K<sup>+</sup>-buffer) as described above with the addition of 50 mM CF. Following the  
12 extrusion step, the vesicle suspension was applied to Sephadex G-50 spin columns  
13 equilibrated with K<sup>+</sup>-buffer to remove free CF. The final phospholipid concentration was  
14 determined by phosphate analysis according to Rouser *et al.* [30]. Vesicles were resuspended  
15 in K<sup>+</sup>-buffer to a concentration of 25 μM and 5b was added at the desired concentration 1 min  
16 prior to the addition of nisin A (50 nM, final conc.). High-salt experiments were conducted in  
17 K<sup>+</sup>-buffer supplemented with 0.5M NaCl. The nisin-induced CF leakage from the vesicles  
18 was monitored with excitation and emission wavelengths set at 430 nm and 513 nm,  
19 respectively. Triton X-100 was added 1 min after the addition of nisin, to a final concentration  
20 of 0.2% (w/v) to fully disrupt the lipid vesicles and the corresponding fluorescence was taken  
21 as 100% leakage. The 5b-induced reduction of leakage is expressed as a percentage of the  
22 leakage observed in the absence of 5b relative to the total release of carboxyfluorescein after  
23 addition of Triton X-100. The leakage results in an increase of fluorescence as the dye self-  
24 quenching is reduced.

## 1 2.11. Membrane depolarization assay

2 The cytoplasmic membrane depolarization activity of 5b was determined with the membrane  
3 potential-sensitive dye DiSC<sub>2</sub>(5) using *Staphylococcus simulans* and *Micrococcus flavus*  
4 grown in LB broth at 37°C and 30°C, respectively. Bacterial cells in the mid-logarithmic  
5 phase were centrifuged at 5000 rpm, washed in 5 mM HEPES (pH 7.8), and resuspended in  
6 the same buffer to an optical density at 600 nm of 0.05 in a 1 cm cuvette. A stock solution of  
7 DiSC<sub>2</sub>(5) was added to a final concentration of 0.4 μM and quenching was allowed to occur at  
8 room temperature for approximately 1 min. The desired concentration of 5b was added and  
9 changes in fluorescence due to the disruption of the membrane potential gradient across the  
10 cytoplasmic membrane were continuously recorded with a Cary Eclipse spectrofluorometer at  
11 an excitation and emission wavelengths of 622 and 670 nm, respectively.

## 14 3. RESULTS

### 16 3.1. Structure-based virtual screening and identification of GT inhibitors hits

17 We set out using a virtual screening approach based on the published *S. aureus* PBP2  
18 structure in complex with moenomycin (PDB entry: 2OLV) [5] to identify small molecule  
19 inhibitors of the GT activity and to reduce the number of compounds to be tested using a  
20 direct *in vitro* assay based on lipid II substrate (see Materials and Methods). This approach  
21 has been used successfully in the identification of inhibitors of other targets of the cell wall  
22 synthesis pathway [31, 32].

23 The selected compounds were first evaluated with *E. coli* PBP1b then with other GT  
24 including *S. aureus* PBP2 [5, 17]. In the first step of screening, the 30 highest ranked  
25 compounds (predicted pK<sub>d</sub> values between -3.61 and -4.37) were selected for biochemical

1 testing. The number of compounds tested was further reduced to 21 as the remaining  
2 compounds were insoluble. Soluble compounds were then tested *in vitro* at 0.2 and 1 mM  
3 final concentrations for their ability to inhibit *E. coli* PBP1b GT activity in a 96-well  
4 microtiter plate assay with fluorescent lipid II. Compounds which inhibited PBP1b activity or  
5 interfered with the fluorescence assay were additionally tested in a radioactivity-based assay.  
6 One compound (5, ranked as the fifth best by eHits) (Figure 1A), a 2-[1-[(2-  
7 chlorophenyl)methyl]-2-methyl-5-methylsulfanylidol-3-yl]ethanamine, was found to inhibit  
8 the GT activity of *E. coli* PBP1b (IC<sub>50</sub> of 59 μM). In the second step, a search based on two-  
9 dimensional similarities to compound 5 identified an analogue of compound 5 called 5b  
10 (Figure 1A), about 2 times more active than compound 5 against *E. coli* PBP1b (IC<sub>50</sub> of 29  
11 μM) and was the focus of further characterisation.

12 The eHiTS-predicted binding mode of compound 5b is presented in Figure 1B. The  
13 model shows that compound 5b partly overlaps with co-crystallized moenomycin rings C and  
14 E, but is buried deeper because of favourable hydrophobic interactions. On the other hand  
15 moenomycin does not form hydrophobic interactions in the hydrophobic shelf due to its  
16 hydrophilic character.

17 Interestingly, some indole derivatives were shown to inhibit lysozyme, a structural  
18 homologue of GT [33]. The docking model of indole 3-carbinol into the active site of  
19 lysozyme [34] shows some similarities with the predicted binding of compounds 5/5b to the  
20 GT. Compounds 5/5b at 1mM concentrations do not inhibit lysozyme and have no effect on  
21 the penicillin-binding activity of the second domain of *E. coli* PBP1b or the *Actinomadura*  
22 R39 DD-peptidase (data not shown). This indicates that compounds 5 and 5b do not exhibit a  
23 promiscuous behaviour. Two other assays based on the use of a detergent [35, 36] and  
24 aggregation of the green fluorescent protein (GFP) [37] also support this observation (data not  
25 shown).

### 1 3.2. Evaluation of compounds 5 and 5b for GTs inhibition and antibacterial activity.

2 Both 5 and 5b were able to inhibit the GT reaction catalyzed by the five GTs tested. The IC<sub>50</sub>  
3 values of compound 5b for *E. coli* PBP1b, *S. aureus* PBP2 and MtgA, *T. maritima* PBP1a and  
4 *E. hirae* PBP2 were determined using the fluorescence assay and were in the micromolar  
5 range (21-56  $\mu$ M) (Table 1).

6 Furthermore, both 5 and 5b exhibit antibacterial activity against several important  
7 human Gram-positive pathogens with MIC values as low as 4  $\mu$ g/ml (Table 2). Interestingly  
8 the compounds displayed MIC values of 4-8  $\mu$ g/ml against methicillin-resistant *S. aureus*  
9 strains (MRSA) which pose a major problem in hospitals. The two GT inhibitors were less  
10 active against Gram-negative bacteria, as observed for other indole and tryptamine containing  
11 compounds [38-40]. The MIC values of compounds 5 and 5b against *E. coli* 1411 were 64  
12 and 16  $\mu$ g/ml respectively. They decreased to the values observed in Gram-positive organisms  
13 after polymyxin B nonapeptide (PMBN) treatment, or in an *acrAB* efflux mutant (Table 2).  
14 These data suggest that uptake of the compounds into *E. coli* is affected both by efflux and  
15 poor permeation through the outer membrane.

16 The effect of different concentrations of compound 5b on the growth and survival of *S.*  
17 *aureus* was evaluated by measuring the number of living cells and turbidity over time. The  
18 killing curves at 1x MIC (10  $\mu$ M) and 2x MIC show that 5b causes an approximately 1 log<sub>10</sub>  
19 and 1.3 log<sub>10</sub> reduction of viability respectively after 90 min (Figure 2). At time zero (during  
20 sample mixing) the cell count was slightly lower than the control. At 4x MIC, the killing  
21 curve shows that 5b causes an immediate decrease in the bacterial count, more important than  
22 that observed at 1x and 2x MIC (1x log<sub>10</sub> approximately), followed by a slower phase yielding  
23 an approximately 5.4 log<sub>10</sub> reduction of viability after 90 minutes (Figure 2) and no viable  
24 cells were observed after 120 min (data not shown) showing that the compound is  
25 bactericidal. Similarly, the cell turbidity (Absorbance at 600nm) at 4x MIC also decreased

1 (36%) immediately after addition of 5b, then remained unchanged (data not shown). It seems  
2 that a two-phase process is taking place upon addition of 5b to the cells: a rapid phase occurs  
3 immediately after addition of the compound followed by a slower one.

### 5 **3.3. Mode of action of compounds 5 and 5b**

#### 6 **3.3.1. Compounds 5 and 5b induce cell wall biosensors in *Bacillus subtilis*.**

7 Compounds 5 and 5b were predicted to specifically target peptidoglycan synthesis in bacterial  
8 cells. In order to characterize their mode of action we have employed a *Bacillus subtilis*  
9 reporter assay which differentiates inhibitors of cell wall, DNA, RNA, protein and fatty acid  
10 biosynthesis by quantifying the upregulation of specific luciferase reporter gene constructs in  
11 each pathway [24, 25]. Using this system, only the cell wall biosensor expression was induced  
12 in the presence of compounds 5 and 5b showing that they specifically inhibit cell wall  
13 biosynthesis as initially hypothesised (Table 3).

14 Two possible mechanisms for inhibition of the GT reaction include direct binding to  
15 the enzyme, or binding to the lipid II substrate. Experiments using fluorescence and  
16 microcalorimetry were not conclusive on whether 5b binds a GT enzyme (data not shown).  
17 Therefore, we checked whether 5 and 5b could interfere with the lipid II. First we used the  
18 *Bacillus subtilis* reporter strain BFS2470 ( $\beta$ -galactosidase reporter system) sensing cell wall  
19 antibiotics that interfere with the lipid II cycle [26, 27]. Using a disk diffusion assay with  
20 different antibiotics or compounds 5 or 5b on agar-plates containing X-Gal, the following  
21 observations were made (Figure 3A): compounds 5, 5b and nisin (as a positive control)  
22 produced a blue colour at the edge of the inhibition halo indicating an interaction of  
23 compounds 5 and 5b with the lipid II. In contrast, moenomycin and ampicillin did not induce  
24  $\beta$ -galactosidase and the blue colour around the inhibition zone. These results indicate that  
25 compounds 5 and 5b could interact with lipid II.



### 1 **3.3.2. Compound 5b interacts with lipid II.**

2 To test for a possible specific interaction between 5b and lipid II, a competition assay was set  
3 up with the lantibiotic nisin, which uses lipid II for its pore-forming activity [14, 41, 42]. This  
4 experiment was performed with compound 5b which is more active than 5 on GT activity. A  
5 similar approach had previously been used with vancomycin in the study that determined the  
6 inhibition of the lipid II-dependent pore-forming activity of nisin by vancomycin in intact  
7 cells [43] and in a model membrane setup as is used here (data not shown) [44]. The addition  
8 of nisin to carboxyfluorescein-loaded vesicles containing lipid II causes leakage of the  
9 fluorescent dye from the vesicles, due to lipid II-dependent pore formation by nisin (CF  
10 loaded vesicles without lipid II showed negligible leakage after adding nisin, Figure 4). When  
11 50 nM nisin was added to 25  $\mu$ M DOPC vesicles containing 0.1 mol% lipid II, a leakage of  
12 ~75% was observed. In the presence of 2.5  $\mu$ M 5b, CF leakage was significantly decreased by  
13 ~36%. Moreover, increasing concentrations of 5b resulted in higher reductions and almost  
14 complete inhibition of leakage at 15  $\mu$ M (1.5x MIC) (Figure 3B). These results suggest that  
15 5b binds to lipid II, thereby preventing an interaction of the latter with nisin.

16 To get insight into the binding site of 5b on lipid II, we first tested whether 5b could  
17 also inhibit the lipid I-dependent pore-forming activity of nisin. Nisin does not discriminate  
18 between lipid I (lacking the GlcNAc moiety) and lipid II. Leakage due to pore formation of  
19 nisin with lipid I was diminished to an equal extent by the presence of 5b as with lipid II (data  
20 not shown). This suggests that the GlcNAc moiety of lipid II is not an essential requirement  
21 for the interaction. If 5b specifically interacts with lipid II, structurally related compounds  
22 should compete for binding. In order to test possible interaction of 5b with the  
23 (pyro)phosphate moiety of lipid II, the inhibition effect of 5b on nisin-lipid II pore formation  
24 was analyzed in the presence of the peptidoglycan biosynthetic intermediates  
25 undecaprenylphosphate (11-P) or undecaprenylpyrophosphate (11-PP), respectively (Figure

1 3C). Nisin activity in the presence and absence of 5b was analyzed in CF-loaded DOPC  
2 vesicles containing 0.1 mol% lipid II and 2 mol% of either 11-P, 11-PP or, as a control, the  
3 anionic lipid DOPG. Strikingly, in the 11-PP containing vesicles, nisin induced CF-release  
4 was not significantly reduced in the presence of 2.5  $\mu\text{M}$  5b. In comparison, 5b was still able to  
5 cause significant inhibition of nisin activity in the presence of 11-P or DOPG, albeit  
6 somewhat less profound than in their absence (Figure 3C). This indicates that the excess of  
7 11-PP competes with lipid II for binding 5b and that the pyrophosphate moiety is important  
8 for the interaction. To investigate this in more detail, NMR measurements were performed  
9 using undecaprenylpyrophosphate in detergent micelles in the presence and absence of 5b.  $^{31}\text{P}$   
10 NMR revealed a change in chemical shift ( $\Delta\delta\text{P}=-0.4$  ppm) for one of the phosphate groups in  
11 the presence of 5b (Figure 3D). No significant shifts were observed when 5b was added to  
12 undecaprenylphosphate-containing Triton-X100 micelles (data not shown). These results  
13 confirm the importance of the pyrophosphate moiety for the interaction of 5b with lipid II.

14

### 15 **3.3.3. Membrane disruptive effects of 5b**

16 The vesicle binding assay using ultracentrifugation showed that 5b was present exclusively in  
17 the pellet in both lipid II-containing and pure DOPC large unilamellar vesicles (LUVs) while  
18 5b alone was present in the supernatant (data not shown). This shows that 5b has an affinity  
19 for the lipid vesicles, regardless of whether lipid II is present or not. To gain insight into the  
20 effect of 5b on membrane, leakage experiments were subsequently conducted with LUVs  
21 containing anionic phospholipids in the absence of lipid II. Vesicles composed of only the  
22 zwitterionic lipid DOPC displayed negligible leakage even in the presence of 40  $\mu\text{M}$  5b  
23 (Figure 4A). Strikingly, in the presence of the negatively charged phospholipid DOPG  
24 leakage was detected at 5  $\mu\text{M}$  concentration and almost total CF-leakage was obtained at 20  
25  $\mu\text{M}$  of 5b (Figure 4A). This suggests that 5b has a general membrane effect independent of

1 lipid II, and this disruption of membrane integrity is anionic lipid-dependent.

2 To investigate if 5b exerts similar effects on bacterial membranes that are known to  
3 contain relatively large amounts of negatively charged lipids, the depolarization effect of 5b  
4 on the plasma membrane in intact cells of *Staphylococcus simulans* and *Micrococcus flavus*  
5 was determined using the membrane-potential sensitive fluorophore DiSC<sub>2</sub>(5). This dye  
6 inserts into the cytoplasmic membrane in the presence of a membrane potential resulting in  
7 self-quenching of fluorescence. The addition of 5b to a final concentration of 1 μM caused a  
8 major release of the dye from the membrane in *M. flavus* (Figure 4b) showing that the  
9 membrane potential was dissipated upon binding of 5b. *S. simulans* also showed significant  
10 membrane depolarization at 5μM concentration of 5b. Nisin was more effective in dissipating  
11 the membrane potential in *M. flavus* (Figure 4b), which is in relative agreement with the  
12 difference in MIC values for both compounds (~5 nM nisin for *M. flavus* versus ~10 μM 5b  
13 for most Gram-positive strains tested).

14 These results are supported by experiments performed with the LIVE/DEAD  
15 BacLight™ bacterial viability assay [45]. The membrane damage inflicted by a 10 minute  
16 exposure to compound 5b on *S. aureus* was analysed. Cells treated with the test agent 5b at 4x  
17 MIC only maintained 33% membrane integrity (67% damage), which does suggest that the  
18 membranes of *S. aureus* cells were permeabilized by the addition of 5b.

#### 19 20 **4. DISCUSSION**

21  
22 Peptidoglycan assemblage can be efficiently disrupted either through the inhibition of  
23 the glycosyltransferase or the transpeptidase enzymatic activities or by sequestration of the  
24 lipid II, thus, preventing its use as a substrate.

25 In our effort to identify small molecules inhibitors of the GT reaction, we have

1 discovered two structurally related indole derivative compounds (5 and 5b) inhibiting several  
2 GTs in vitro and having good antibacterial activity in the micromolar range. These  
3 compounds specifically induce peptidoglycan pathway stress sensors in *Bacillus*, confirming  
4 that bacterial cell wall synthesis is the target.

5 Moreover, we have found that compound 5b binds to the lipid II precursor. This is  
6 reminiscent of several lipid II-targeting antibiotics such as vancomycin and related (second  
7 generation) glycopeptides [46], and the lantibiotics mersacidin and nisin [11].  
8 Undecaprenylpyrophosphate competes with lipid II for interaction with 5b. As  
9 undecaprenylphosphate and DOPG did not significantly compete with lipid II for 5b, the  
10 pyrophosphate moiety appears to be essential for the interaction with 5b. Nisin also binds the  
11 pyrophosphate of lipid II, and this could explain the effective inhibition of nisin-lipid II pore  
12 assembly by 5b. The data obtained from NMR analysis of undecaprenylpyrophosphate in the  
13 presence and absence of 5b further strengthens the hypothesis that the pyrophosphate moiety  
14 is critical for complex formation of 5b with lipid II. The amine residue of 5b has  
15 independently been shown to be essential for its glycosyltransferase inhibiting activity,  
16 suggesting its crucial involvement in the interaction with the pyrophosphate group of lipid II.  
17 The bacitracin antibiotic has also been shown to inhibit PG synthesis by sequestration of  
18 undecaprenyl pyrophosphate and was known to specifically bind to the pyrophosphate moiety  
19 of the lipid carrier [47].

20 Compound 5b at low concentration caused membrane depolarization in *M. flavus* and  
21 *S. simulans* as did nisin. Several lines of evidence also indicate that compound 5b affects in  
22 vitro membrane integrity in a negatively charged lipid dependent way in the absence of lipid  
23 II. These results suggest that membrane disruption could contribute to its antibacterial  
24 activity. Indeed, the effect of 5b on *S. aureus* viability shows that a two phase process  
25 contributes to its bactericidal activity. The first and immediate effect could be the result of

1 membrane integrity disruption. The second and slower phase (4x MIC) is more likely the  
2 consequence of cell wall synthesis inhibition as was previously observed for the kinetics of  
3 inhibition of *S. aureus* by moenomycin [48]. Therefore, it appears from our data that the  
4 identified compounds have two modes of action, namely, inhibition of cell wall synthesis and  
5 membrane depolarization/disruption. This mode of action resembles that of nisin which binds  
6 and sequesters lipid II preventing its use by the peptidoglycan polymerases and inducing  
7 pores formation in the bacterial membrane and membrane depolarization. The properties  
8 described for 5b could also be shared by compound 5 due to close similarity between the two  
9 compounds.

10 Bacterial resistance to antibiotics requires a continuing effort to find new antibacterial  
11 compounds able to cope with emerging resistant strains. The glycosyltransferase step in  
12 peptidoglycan biosynthesis is a validated target for new antibacterial development. The small  
13 antibacterial molecules described here are much simpler agents compared to known natural  
14 products targeting lipid II. Considering the importance of lipid II and the pyrophosphate  
15 motif for the whole enzymatic machinery of the bacterial cell wall synthesis pathway, this  
16 simple mode of binding to lipid II may limit the occurrence of resistance to such promising  
17 compounds. Finally, this work provides the basis for the development of more active and  
18 specific analogues of compounds 5/5b to target the essential GT step in cell wall synthesis  
19 using small molecule inhibitors.

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## 1 **Figure legends**

2  
3  
4  
5 **Figure 1. Structures of the compounds 5 and 5b and the predicted binding model of**  
6 **compound 5b to *S. aureus* PBP2 active site. A.** Structures of the compounds 5, 2-[1-[(2-  
7 chlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine and 5b, 2-[1-[(3,4-  
8 dichlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine. **B.**  
9 Superimposition of the computer model of compound 5b (blue) on the X-ray structure of  
10 moenomycin (magenta lines, moenomycin rings are labeled from A to F) bound to *S. aureus*  
11 PBP2 (pdb code: 2OLV). Amino acids that interact with compound 5b are presented as sticks.  
12 Hydrophobic residues are shown as semi-transparent yellow surfaces. Hydrogen bonds  
13 between the free amino group of compound 5b and the Asp156 side chain are represented as  
14 dotted lines.

## 14 **Figure 2. Effect of compound 5b on bacterial viability.**

15 Cell viability after exposure of *S. aureus* ATCC 25923 to 1, 2, and 4 times the MIC of  
16 compound 5b.

## 18 **Figure 3. Interaction of compound 5 and 5b with lipid II.**

19 **A.** Effect of compounds 5, 5b and control agents on the lipid II cycle. The control antibiotics  
20 or compounds were spotted at the indicated amount on a Whatman disc on an agar-plate  
21 containing X-Gal and inoculated with the *Bacillus subtilis* strain BFS2470. The picture was  
22 taken after overnight incubation at 37°C.

23 **B.** Effect of 5b on nisin pore formation in DOPC LUVs containing 0.1 mol % lipid II.

24 Compound 5b reduced the leakage of carboxyfluorescein induced by nisin.

25 **C.** Competition of undecaprenylpyrophosphate (11-PP), undecaprenylphosphate (11-P) and

1 DOPG with lipid II (LII) (20 fold molar excess over lipid II) for 5b (2.5  $\mu$ M) binding. The  
2 averages of two independent experiments are shown with the error bars indicating the spread  
3 between values. Experiments were also conducted in the absence of 5b as the leakage  
4 reduction is depicted as a percentage of the leakage without 5b (the latter was similar in all  
5 three cases).

6 **D.**  $^{31}$ P-NMR of undecaprenylpyrophosphate in Triton-X100 micelles in the presence or  
7 absence of 1 mM 5b.

#### 9 **Figure 4. Membrane disruption effect of compound 5b.**

10 **A.** Comparison of the disruptive effect of compound 5b on DOPC/DOPG (1:1 mol/mol) and  
11 DOPC vesicles.

12 **B.** Effect of 5b on the plasma membrane potential of *Staphylococcus simulans* and  
13 *Micrococcus flavus* using DiSC<sub>2</sub>(5). Dye release was monitored at excitation and emission  
14 wavelengths of 622 and 670 nm, respectively.

#### 17 **List of abbreviations**

18 PBP: penicillin-binding protein

19 GT: glycosyltransferase

20 PG: peptidoglycan

21 CF: carboxyfluorescein

22 DOPC: 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine

23 DOPG: 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]

24 11-PP: undecaprenylpyrophosphate

25 11-P: undecaprenylphosphate

26 LUV: large unilamellar vesicle

**Table 1.** IC<sub>50</sub> values of compound 5b for various GTs.

IC <sub>50</sub> (μM)	
	Compound 5b
<i>E. coli</i> PBP1b	29 ± 12
<i>S. aureus</i> PBP2	20.7 ± 1
<i>T. maritima</i> PBP1a	56 ± 7
<i>E. hirae</i> PBP2	33 ± 6
<i>S. aureus</i> MtgA	27 ± 9

The IC<sub>50</sub> of compound 5 for *E. coli* PBP1b was 59 ± 3.3 μM. For comparison, the IC<sub>50</sub> value of moenomycin for *S. aureus* PBP2 was 1.3 ± 0.3 μM.

**Table 2.** Susceptibility of a panel of bacteria to compounds 5, 5b and moenomycin.

	MIC ( $\mu\text{g/ml}$ )		
	5	5b	moenomycin
<i>E. faecalis</i> ATCC 29212	16	4 (10 $\mu\text{M}$ )	4 (0.6 $\mu\text{M}$ )
<i>L. innocua</i> ATCC 33090	8	4	4
<i>S. aureus</i> ATCC 25923 (MSSA)	8	4	0.5
<i>S. aureus</i> ATCC 43300 (MRSA)	8	4	0.5
<i>S. aureus</i> PL1 (MRSA)	8	4	0.5
<i>M. luteus</i> ATCC 9341	8	4	> 64
<i>S. epidermidis</i> ATCC 12228	8	4	4
<i>S. pneumoniae</i> D39	16	4	nd
<i>E. coli</i> 1411	64	16	nd
<i>E. coli</i> 1411 + PMBN (4 $\mu\text{g/ml}$ )	16	8	nd
<i>E. coli</i> SM1411 ( $\Delta\text{acrAB}$ )	16	4	nd
<i>Pseudomonas aeruginosa</i>	> 250	> 250	nd

PMBN, polymyxin B nonapeptide. nd, not determined.



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**Table 3.** Induction of *B. subtilis* antibiotic biosensors in response to a panel of antimicrobial agents.

Antimicrobial Agent	Upregulated biosynthetic pathway				
	<i>Cell Wall</i>	<i>Protein</i>	<i>RNA</i>	<i>DNA</i>	<i>Fatty Acid</i>
Vancomycin	+	-	-	-	-
Flucloxacillin	+	-	-	-	-
<b>5 or 5b</b>	+	-	-	-	-
Nisin	-	-	-	-	-
Mersacidin	+	-	-	-	-
Cetyltrimethylammonium bromide	-	-	-	-	-
Tetracycline	-	+	-	-	-
Anhydrotetracycline	-	+	-	-	-
Fusidic Acid	-	+	-	-	-
Rifampicin	-	-	+	-	-
Rifamycin SV	-	-	+	-	-
Ciprofloxacin	-	-	-	+	-
Trimethoprim	-	-	-	+	-
Triclosan	-	-	-	-	+

+/- Induced/uninduced biosensor in response to a range of concentrations of antimicrobial agent (0.005-20µg/ml).

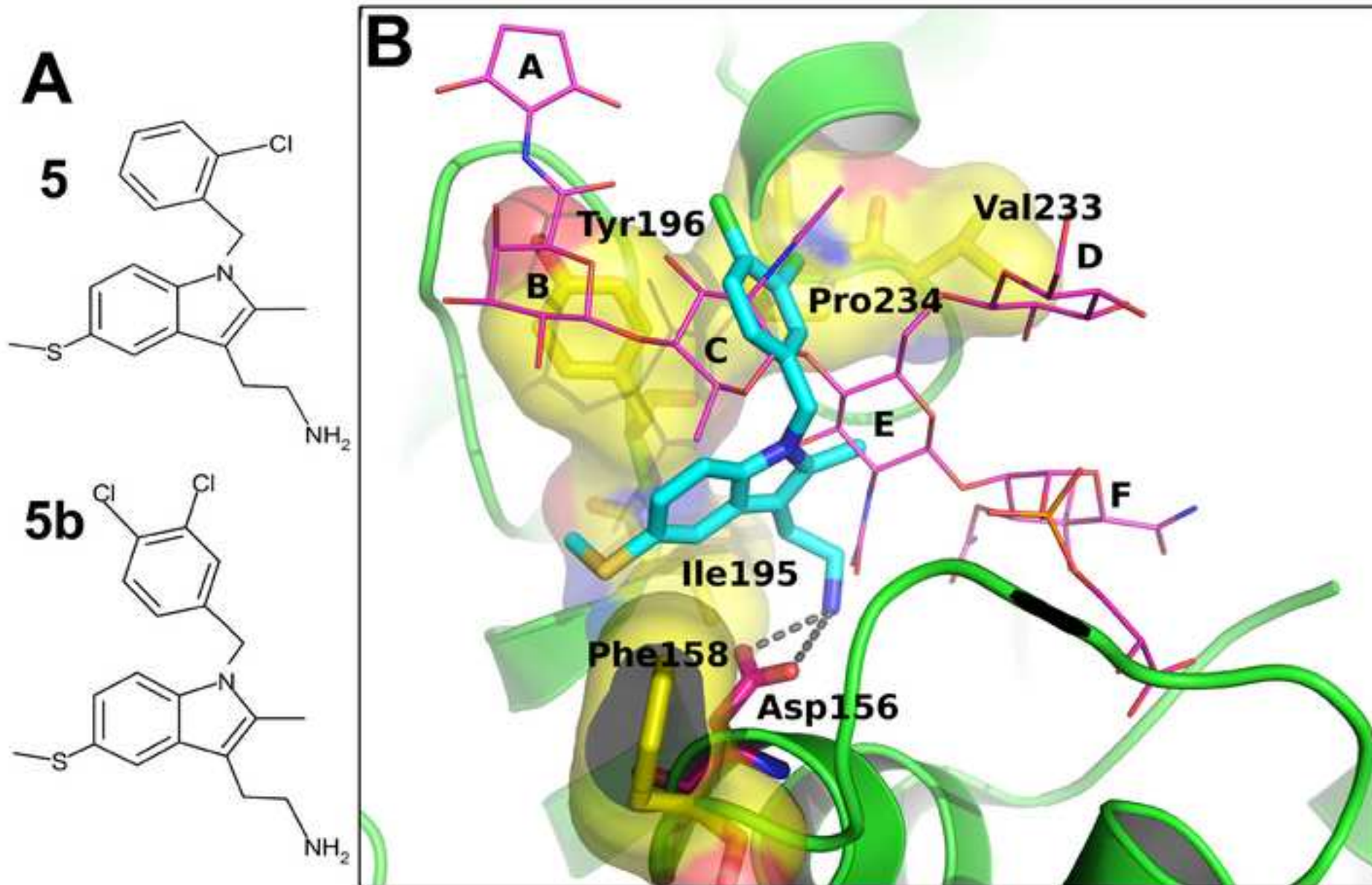


Figure2  
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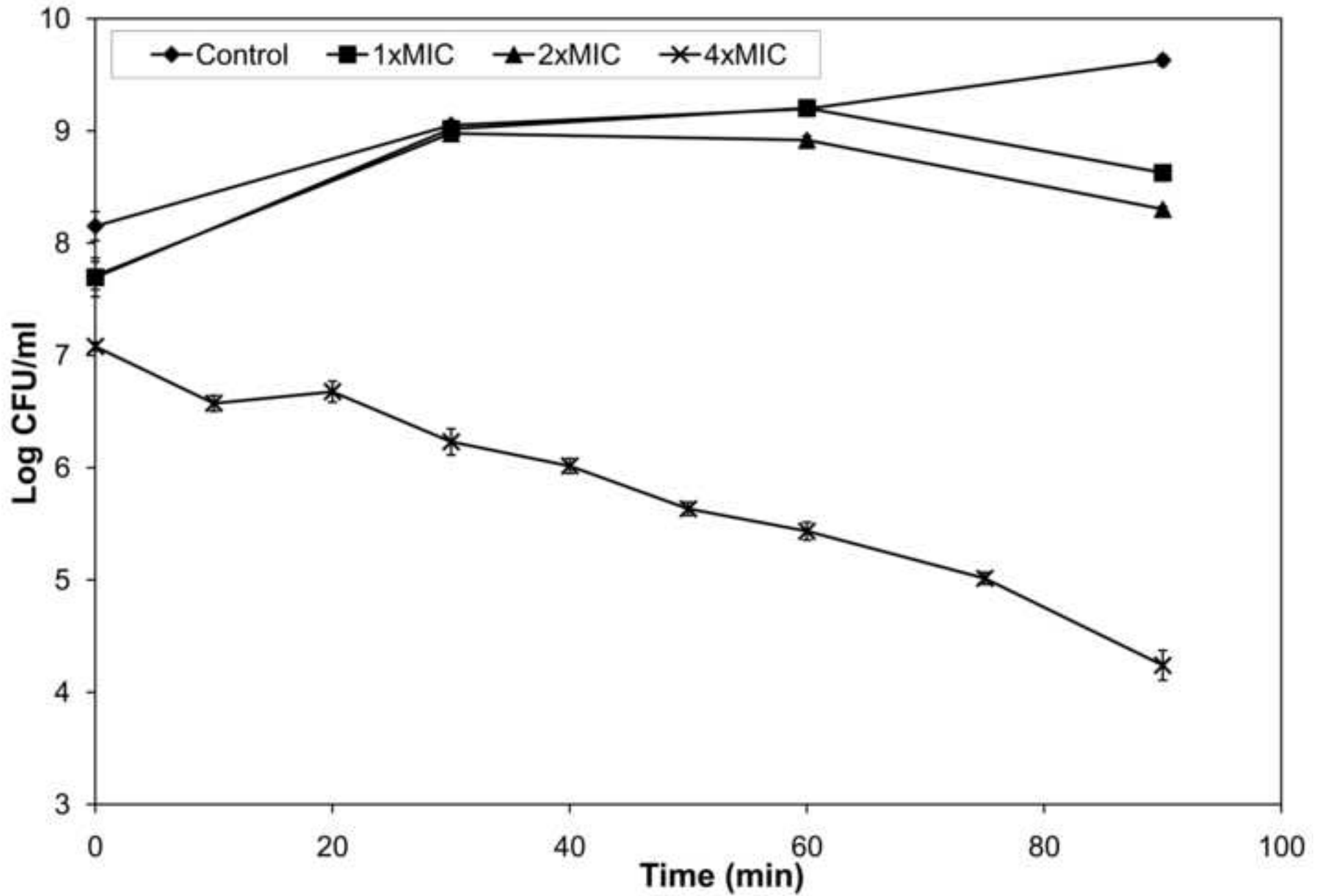


Figure3

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trip

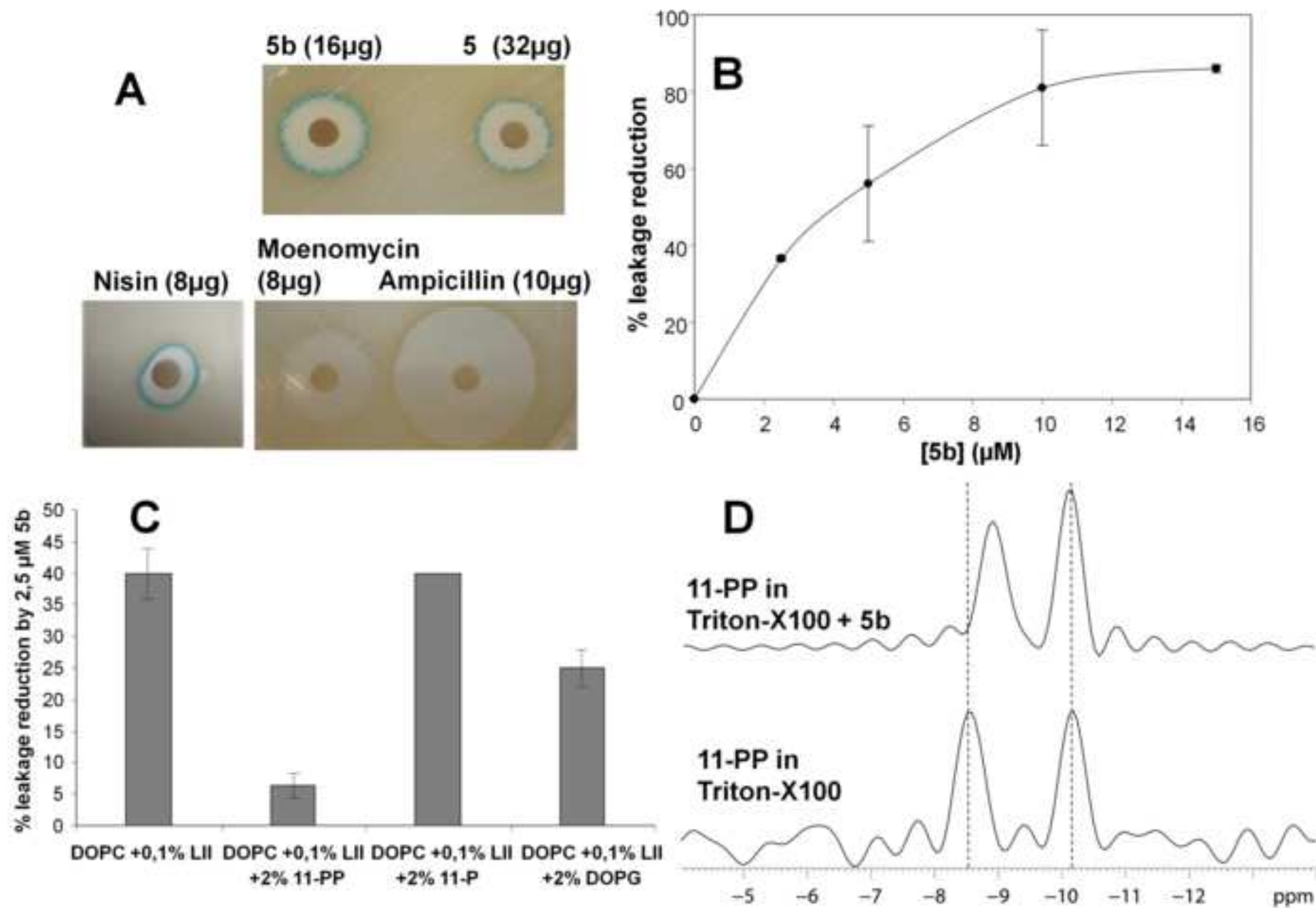


Figure4

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