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Effects of the Selected Iminosugar Derivatives on Pseudomonas aeruginosa Biofilm Formation

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Effects of the selected iminosugar derivatives on *Pseudomonas aeruginosa* biofilm formation

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Keyword:	Antimicrobial, Microbial Drug Resistance, Bacteria
Abstract:	A lack of an effective way to eliminate pathogenic bacteria hidden in the biofilm is a major problem in treatment of chronic bacterial infections. Iminosugar derivatives are potential candidates for inhibitors of enzymes taking part in the biosynthesis of exopolysaccharides, which are forming bacterial biofilm. Investigated iminosugars were studied either at an early stage of biofilm formation or later on when the mature biofilm of <i>Pseudomonas aeruginosa</i> was already formed. A series of diverse iminosugar structures significantly inhibited biofilm formation, whereas, they showed no influence on already formed biofilm. This indicates a possible mechanism of their action based on inhibition of exopolysaccharide backbone synthesis in the early stages of biofilm formation. Moreover, iminosugar derivatives did not show significant effect on the viable bacteria numbers in both early and mature biofilm forms. Importantly, they were not cytotoxic against human Caco-2 cells in vitro, which may be to their advantage in case of their medical application in preventing <i>P. aeruginosa</i> biofilm formation.

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3 **Effects of the selected iminosugar derivatives on *Pseudomonas aeruginosa* biofilm**
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5 **formation**
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52 **Running head: Iminosugars inhibit *Pseudomonas* biofilm**
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3 A lack of an effective way to eliminate pathogenic bacteria hidden in the biofilm is a major
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5 problem in treatment of chronic bacterial infections. Iminosugar derivatives are potential
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7 candidates for inhibitors of enzymes taking part in the biosynthesis of exopolysaccharides,
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26 their advantage in case of their medical application in preventing *P. aeruginosa* biofilm
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28 formation.
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35 Introduction

36
37 Iminosugars, structural analogues of carbohydrates in which the oxygen atom is replaced by
38
39 nitrogen, have been known since the 60's with the discovery of nojirimycin and related
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41 compounds.¹⁹ However, natural iminosugars as well as their synthetic derivatives have been
42
43 recently found to exhibit a wide range of biological activities toward carbohydrate-dependent
44
45 enzymes, most prominently glycosidase inhibitors.^{18,25} Studies on the effect of iminosugars on
46
47 various cellular functions have been reported by several groups.^{7,18} Moreover, they are
48
49 currently under investigation as potential therapeutic agents in many human disorders
50
51 including cancer, diabetes, lysosomal storage disorder (*i.e.* Gaucher disease), HIV infection,
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53 hepatitis C virus infection and others.^{7,28}
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3 Iminosugar derivatives appear also to be interesting candidates as potential inhibitors
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5 of glycosyltransferases involved in the biosynthesis of glycan chains,⁶ or of more specialized
6
7 enzymes such as UDP-Galactopyranose mutase or UDP-Galactofuranose transferases.²³ The
8
9 latter are involved in the formation of galactofuranose units and production of Galf-containing
10
11 polysaccharides.^{10,21} This unusual form of galactose is found in the exopolysaccharides of
12
13 several bacteria such as *Escherichia coli* K-12, *Streptococcus pneumoniae*, but also in
14
15 *Lactobacillus* species.^{12,14} So far very few studies have been dedicated to the effect of
16
17 iminosugars on bacterial biofilms: a brief report by a Chinese group indicates that
18
19 iminosugars may be useful in caries prevention by affecting α -glucan biosynthesis²⁹ and
20
21 another recent study has provided evidence for a significant inhibitory action of 1-
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23 deoxynojirimycin (from mulberry leaves) on *Streptococcus mutans* biofilm formation.^{13,26}
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29 Biofilm is usually defined as surface-associated microbial communities, surrounded by
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31 an extracellular polymeric substance (EPS)-matrix, comprising mainly polysaccharides,
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33 proteins, nucleic acids, and metabolites produced by bacteria or fungi. The biofilm produced
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35 by microorganisms enables acquisition of new ecological niches and moreover bacteria
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37 hidden in a biofilm matrix are highly resistant to a variety of antibiotics. Therefore, the so-
38
39 called pathogenic biofilm is considered as one of the most important and difficult to cure form
40
41 of the microbial pathogenicity.^{11,24,26}
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46 *Pseudomonas aeruginosa* (PAR) is a bacterial species causing important human
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48 infections. PAR is also responsible for biofilm-related infections like as pneumonia in cystic
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50 fibrosis patients and it is associated with chronic wounds in patients with chronic diabetic foot
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52 infection. Until recently, it has been thought that the main elements of the *P. aeruginosa*
53
54 biofilm structures are alginate polysaccharides. However, at present it is considered that the
55
56 building of the three-dimensional structure of *P. aeruginosa* biofilm matrix begins from the
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58 synthesis of the extracellular exopolysaccharides composed of chains containing sugars such
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3 as rhamnose, glucuronic acid, galactosamine, and only the next step is to fill this initial
4
5 structure with strongly gelling alginate scaffold.^{3,16,24}
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8 The aim of this study was to evaluate the effect of a series of diverse iminosugar
9 structures to inhibit formation of *P. aeruginosa* biofilm. Additionally, the iminosugars were
10 tested for their ability to influence *P. aeruginosa* populations in early and mature biofilm
11 forms and to cause apoptosis and/or necrosis of the human cells. In our *in vitro* study, the
12 early and mature biofilm forms of *P. aeruginosa* was treated with the investigated
13 iminosugars, which were designed to mimic on one hand hexopyranose structures having a
14 pseudo *gluco* (iminoxylitol) or a pseudo *galacto* configuration (imino-L-arabinitol) as well as
15 two other compounds having a configuration resembling that of a galactofuranose.
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30 **Materials and Methods**

31 *Tested compounds*

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33 Investigated iminosugar derivatives have been synthesized in the Institute of Organic
34 and Analytical Chemistry at University of Orleans: **HDIX** – α -1-C-hexyl-1,5-dideoxy-1,5-
35 imino-D-xylitol, a powerful inhibitor of lysosomal β -glucocerebrosidase;^{8,17} **DDIA** – β -1-C-
36 (2,3-dihydroxypropyl)-1,5-dideoxy-1,5-imino-L-arabinitol,¹ has a *galacto*-like configuration
37 and carries a polar aglycone; **DHMBI** – (1*S*,2*S*,3*S*,5*S*,9*R*)-1,2-dihydroxy-3-hydroxymethyl-5-
38 *p*-methoxybenzylindolizidine,⁵ a close analogue of the natural product *steviamine*, and **PDIA**
39 – β -1-C-propyl-1,4-dideoxy-1,4-imino-L-arabinitol, a pyrrolidine analog of **HDIX**. Chemical
40 structures of the compounds are presented in Fig. 1.
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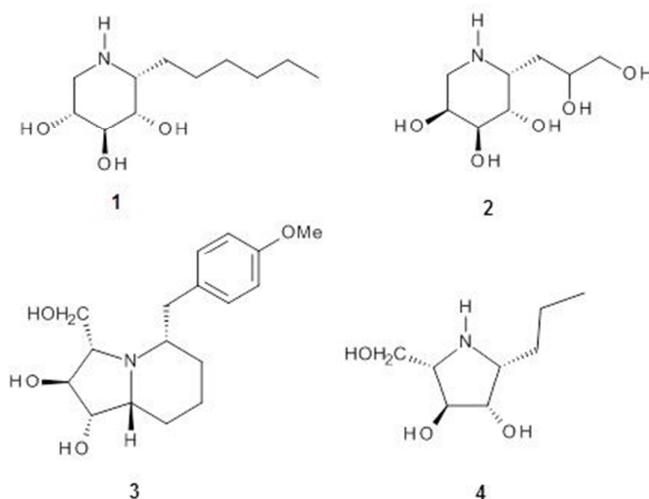


FIG. 1. Chemical structures of the compounds: 1 – **HDIX**, 2 – **DDIA**, 3 – **DHMBI** and 4 – **PDIA**.

The details of the synthesis of **HDIX** and **DDIA** have been recently published.¹ The synthesis of **DHMBI** has also been recently reported,⁵ and **PDIA** was obtained by hydrogenation of the precursor of **DHMBI** (Fig. 2).

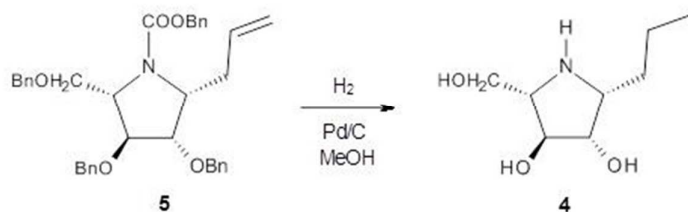


FIG. 2. The synthesis pathway of **PDIA** (4), obtained by hydrogenation of compound 5.

(1R)-1-C-propyl-1,4-dideoxy-1,4-imino-L-arabinitol (**PDIA**): To a solution of compound 5 (302 mg, 0.52 mmol)²⁶ in isopropyl alcohol (*i*PrOH, 5 ml) was added 1 mol l⁻¹HCl (2 ml) and 10% Pd on charcoal (78 mg). The mixture was placed under an atmosphere of hydrogen and vigorously stirred for 18 h; the catalyst was then removed by filtration through a membrane,

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3 washed with *i*PrOH, and the solution was concentrated under reduced pressure. The crude
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5 product was dissolved in water (5 ml), treated with Amberlite IRA-400 (OH⁻) ion exchange
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7 resin, the resin was filtered and the solvent evaporated to provide homogeneous **PDIA** (79
8
9 mg, 86%). Data for **PDIA**: $R_f = 0.12$ (pet. ether : ethyl acetate 7 : 3); $[\alpha]_D -30.1$ (*c* 0.92,
10
11 MeOH). ¹H NMR (Bruker Avance II NMR spectrometer, 400MHz, CD₃OD): δ 3.84 (d, $J=3.3$
12
13 Hz, 1H, H3), 3.77 (d, $J=3.6$ Hz, 1H, H2), 3.67 (d, $J=4.8$ Hz, 2H, H5), 3.08 (dt, $J=3.6$ Hz,
14
15 6.8Hz, 1H, H1), 2.94-2.91 (m, 1H, H4), 1.62-1.39 (m, 4H, H6, H7), 0.97 (t, $J=7.2$, 3H, H8).
16
17 ¹³C NMR (100MHz, CD₃OD): δ 81.53 (C3), 79.27 (C2), 68.68 (C4), 63.30 (C5), 62.65 (C1),
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19 31.67 (C6), 21.36 (C7), 14.66 (C8). ESI-HRMS: calculated for C₈H₁₈NO₃ [M+H]⁺:
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21 176.12812; found: 176.12819.
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29 *Bacterial cultures*

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32 All tests were performed on three *Pseudomonas aeruginosa* strains: PAR 5, PAR 20,
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34 and PAR 50. The strains were randomly selected from a larger collection of PAR from
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36 clinical materials diagnosed in Chair of Microbiology, Jagiellonian University Medical
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38 College in Krakow. The samples for microbiological analyses were taken from wounds of
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40 patients with chronic diabetic foot infections. The strains were propagated in 10 ml of
41
42 Trypticase-Soy Broth (TSB, Difco) at 37°C for 24 hours under aerobic conditions. Then the
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44 cultures were centrifuged (400×g; 10 min) and washed with 10 ml of saline buffer. Initial
45
46 suspensions of bacteria containing 1×10⁸ colony forming units (CFU)/ml were prepared by
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48 making serial dilutions in saline using the MacFarland's scale. Then numbers of bacteria were
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50 confirmed using a standard viable count method on McConkey Agar (Oxoid) using decimal
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52 dilutions.
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3 *Growth conditions and measurement of biofilm formation by P. aeruginosa*

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6 *P. aeruginosa* biofilm was set-up in sterile plastic 96-well plates with adherent surface
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8 (Greiner Bio-One, USA). Twenty microliter quantities of the bacterial stock suspensions,
9
10 prepared as described above, were added to each well followed with 180 μ l of sterile TSB.
11
12 Final concentration of the bacteria was 1×10^8 CFU/ml. The plates were centrifuged for
13
14 10 minutes at $400 \times g$ to sediment bacteria on the bottom of each well and then incubated for
15
16 different time at 37°C under aerobic conditions.
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20 Biofilm quantity was determined using Congo red dye according to a modified
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22 procedure described by Reuter *et al.*²² Briefly, at different time points of the culture the plates
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24 were centrifuged, the culture medium was gently removed from wells and immediately 200 μ l
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26 of 0.1% Congo red solution was added. The plates were left for 30 minutes at room
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28 temperature and then 10 minutes before the end of staining, the plates were centrifuged for
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30 10 minutes $400 \times g$ and then washed twice with buffered saline to remove unbound dye.
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32 Absorbance was measured at $\lambda = 492$ nm wavelength using spectrophotometer (Awarness
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34 Technology Inc., Palm City, FL, USA). All measurements were performed in triplicates and
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36 mean values \pm SD were calculated.
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43 *Effects of the iminosugars on early and mature P. aeruginosa biofilm and on numbers of*
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45 *P. aeruginosa cells embedded in biofilm structure*

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48 Influence of the iminosugars on *Pseudomonas* cells forming biofilm was observed by
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50 filling wells of a 96-well plate with 20 μ l of freshly made standard suspensions of
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52 *P. aeruginosa* strains containing 1×10^8 CFU/ml and 180 μ l of TSB broth (Oxoid), as
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54 described above. Tested substances (**HDIX**, **DDIA**, **DHMBI** and **PDIA**) were added
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56 immediately after setting-up the bacteria at a final concentration of 0.9 mM. Wells filled with
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3 bacterial suspensions only were used as a control. Then, the plates were gently rotated on
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5 table to distribute an iminosugar and incubated for 48 hours (early biofilm) and for 72 hours
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7 (mature biofilm) at 37°C in aerobic conditions.
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10 Biofilm formation and the number of viable bacteria during early biofilm development
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12 under influence of the iminosugars or in control were checked in parallel rows of the wells in
13
14 the following time intervals: 0, 24, and 48 hours. Biofilm quantity was measured in one row
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16 using Congo red method as described above. At the same time intervals, biofilm content in
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18 each well of the second row was removed and mixed by multiple pipetting and transferred to
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20 sterile tubes. Then decimal dilutions of the bacterial suspension were made in TSB broth,
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22 plated on McConkey Agar (Oxoid), and incubated at 37°C for 24 hours. Numbers of colonies
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24 grown on plates were counted and total numbers of the viable bacteria were calculated as
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26 colony forming units (CFU) per ml.
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30 To determine inhibitory properties of iminosugars against mature biofilm, the
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32 substances were added in 48 hours after adherence of bacteria to the wells in a 96-well plate.
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34 The growth conditions of *P. aeruginosa* were exactly the same, as described above. After the
35
36 addition of iminosugars, the biofilm formation and the number of viable bacteria were
37
38 checked in the following time intervals: 48, 48+8, 48+24 hours. Biofilm quantity and numbers
39
40 of viable *Pseudomonas* cells embedded in mature biofilm were estimated as described above.
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43 All measurements were performed in triplicates and mean values \pm SD were calculated.
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46 47 48 *Biofilm inhibition visualization by SEM* 49

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51 Inhibition of biofilm formation upon incubation with iminosugars was visualized by
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53 scanning electron microscopy (SEM) (TESCAN, Vega 3) according to the procedure
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55 previously described.¹⁵ Briefly, glass coverslips were placed in a 12-well plastic plates and
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57 treated with poly-L-lysine solution (Sigma Aldrich) to enhance bacterial cell adhesion. After
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3 this step, standard suspensions of the used *P. aeruginosa* strains were added to each well
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5 followed with the tested compounds (0.9 mM) and incubated for 0, 24, and 48 hours to
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7 observe early biofilm and for 48, 48+8, 48+24 hours for mature biofilm. Then, 1ml of fixing
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9 buffer (sodium cacodylate (Sigma Aldrich) in 2.5 wt% glutaraldehyde (Sigma Aldrich) and
10
11 0.1M sucrose (Sigma Aldrich) was added to the wells and the coverslips were incubated for
12
13 1.5 h at 37°C. A dehydration procedure was applied to the coverslips removed from the wells
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15 in a gradient of ethanol. Dried coverslips were sputtered with a 20 nm gold layer (Quorum;
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17 Q150R S) to facilitate SEM visualization.
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25 *Detection of apoptosis and necrosis caused by tested compounds by fluorescence microscopy*

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28 The Annexin-V-FLUOS Staining Kit (Roche) was used for the detection of apoptotic
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30 and necrotic cells according to the producer's protocol. This kit uses a dual-staining protocol
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32 in which the apoptotic cells are stained with annexin-V (green fluorescence), and the necrotic
33
34 cells are stained with propidium iodide (PI; red fluorescence). Caco-2 cells (human intestinal
35
36 epithelial cell line) were grown for 24 hours in DMEM (Life Technologies) supplemented
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38 with fetal bovine serum (FBS; Sigma-Aldrich) (10 % CO₂, 37°C, 90 % humidity) to about
39
40 70 % confluence. Then the cells were treated with tested iminosugars with final concentration
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42 of 0.9 mM for 24 hours. At the beginning of experiment and after 8 and 24 hours of
43
44 incubation, fluorescence was observed under fluorescence microscope (Olympus, BX51). The
45
46 cells were viewed under magnification of 400× using filters: TRITC (tetramethylrhodamine)
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48 intended for viewing necrotic cells (550-570 nm) and FITC (fluorescein isothiocyanate)
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50 intended for viewing apoptotic cells (495-519 nm).
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Statistical analysis

Statistical analyses were performed using STATISTICA, version 10 software. Statistical significance values of the groups means for three strains of PAR were evaluated using Student's t-test. The statistical analyses performance were considered significant when $p < 0.05$ and highly significant when $p < 0.01$. Since significant differences among the tested strains for their susceptibility to iminosugars activity to their biofilm formation were observed, activities of these substances *versus* control were calculated for mean values for all tested strains.

Results

While examining the effect of the tested compounds on biofilm quantity produced by three *P. aeruginosa* strains PAR 5, PAR 20, PAR 50, we observed differences between two phases of biofilm formation. When iminosugars were added directly to suspensions of *P. aeruginosa*, which were then allowed to sediment, a distinct inhibition of the early biofilm development was observed for all tested compounds, although at different degrees (Fig. 3).

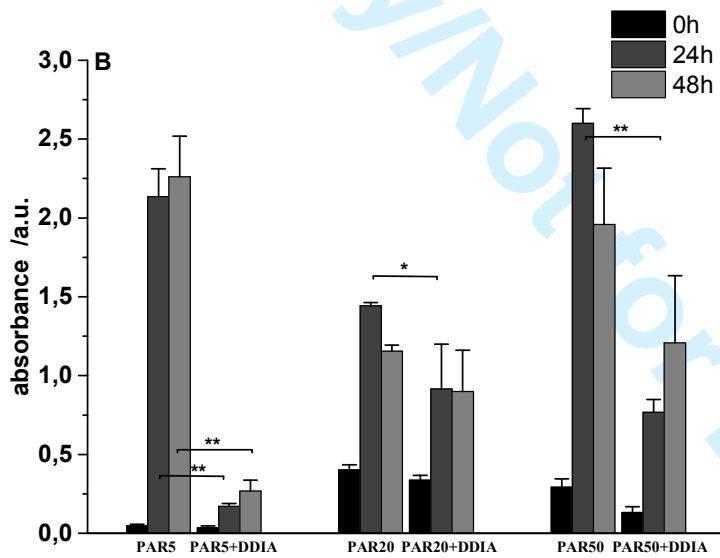
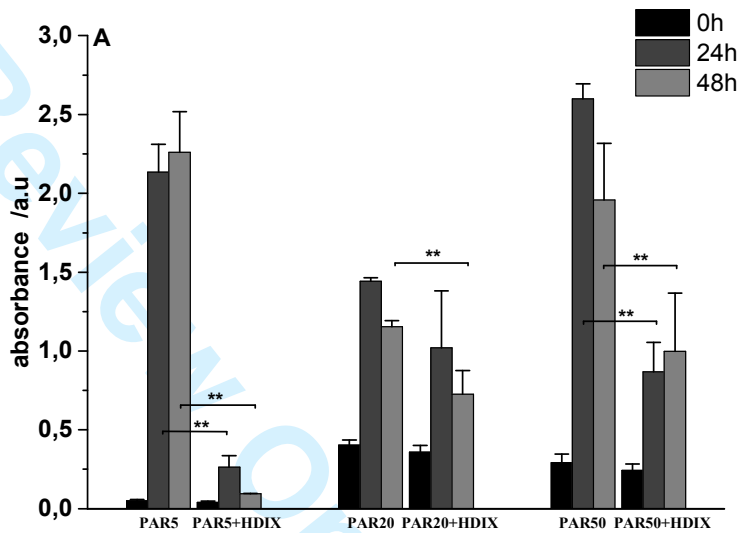
It appeared also, that the tested strains of *P. aeruginosa*, cultured without the iminosugar, differed in the production of the biofilm. The most active were the strains PAR 50 and PAR 5, which after 24 hours liberated largest quantities of biofilm-EPS expressed as absorbance, equal to 2.6 and 2.15, respectively, while the absorbance value of strain PAR 20 was only 1.45.

The compound **DHMBI** appeared to be most active, followed by **HDIX** and **PDIA**, while **DDIA** was less inhibitory. Moreover the latter compound was able to inhibit biofilm production for only 24 hours of incubation.

In addition, such iminosugars as **HDX**, **DHMBI** and **PDIA** showed the most potent anti-biofilm activity, particularly to such strains of *P. aeruginosa* (PAR 5 and PAR 50), in which, after 24 hours, the rate of formation of EPS was the quickest.

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Generally, the biofilm produced by PAR 5 strain was more strongly inhibited than those produced by strain PAR 50 and then by strain PAR 20.



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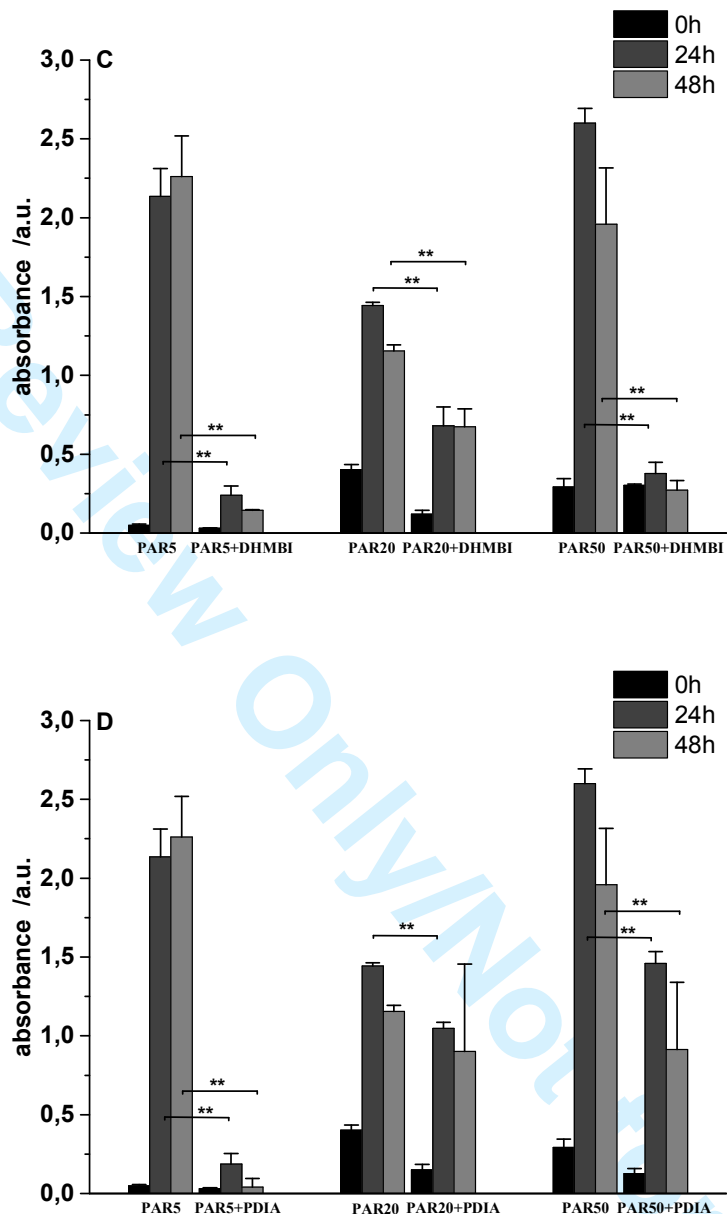
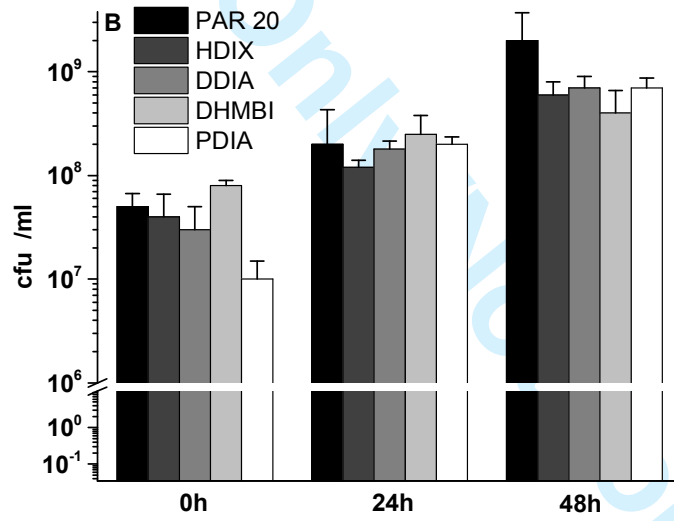
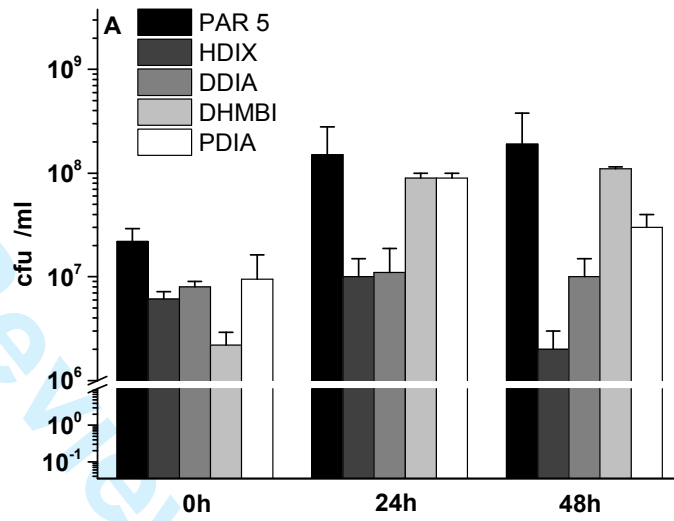


FIG. 3. Effect of the tested iminosugars on PAR cultures in early phase of biofilm formation. (A: HDIX, B: DDIA, C: DHMBI and D: PDIA). Biofilm formation expressed as absorbance at 492 nm after Congo red staining at different time points. Results depict mean values \pm SD calculated from three replicates ($n = 3$). * $p < 0.05$ and ** $p < 0.01$

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3 On the other hand, no significant effect on changes in the viable bacteria numbers was
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5 observed during 48 hours of incubation of the tested PAR with all investigated iminosugars
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7 (Fig. 4). There were also no significant differences between the tested strains.
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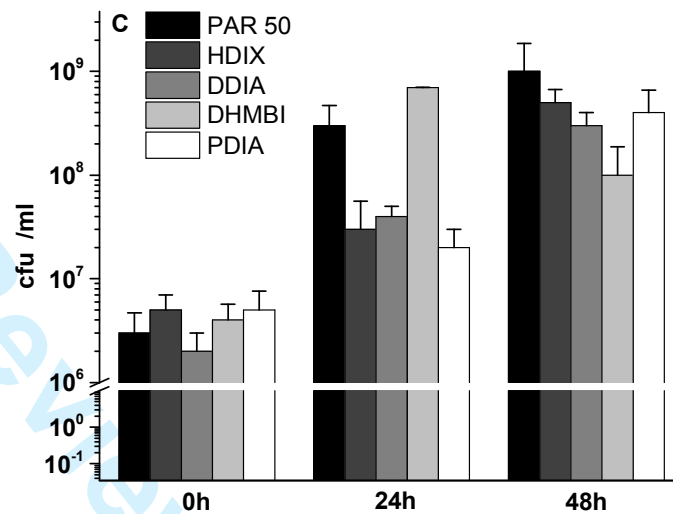
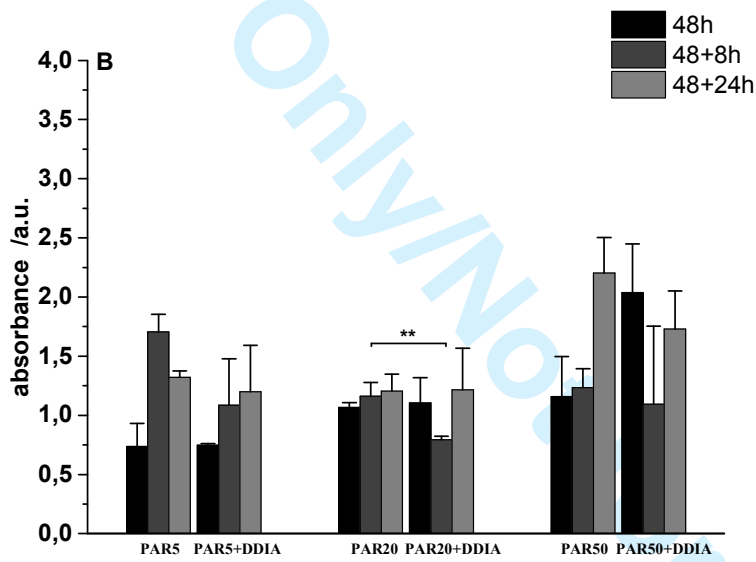
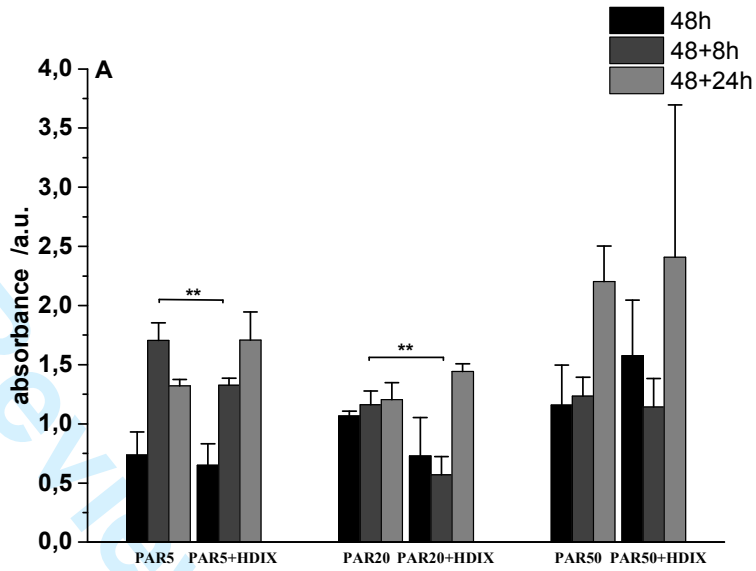


FIG. 4. Effect of the tested compounds on numbers of viable *P. aeruginosa* populations in early biofilm phase formation. Results depict logs of CFU/ml for three *P. aeruginosa* strains (A: PAR 5, B: PAR 20 and C: PAR 50) measured at different time intervals.

A relatively weak influence on biofilm formation was exerted by the iminosugars added to mature biofilm of all tested PAR strains (Fig. 5). The studied compounds were able to inhibit production of *P. aeruginosa* biofilm only up to 8 hours of incubation with already formed biofilm or decompose its already formed structure. The biofilm thickness measured as absorbance after Congo Red staining was almost identical when the strains were incubated for 48+24 hours in the presence of the tested substances or without them. What is noteworthy, only compound **HDIX** showed higher absorbance after staining with Congo red *i.e.* caused increased biofilm amount by the end of the experiment as opposed to three remaining iminosugars for which a slight decrease of the biofilm quantity was observed (Fig. 5).

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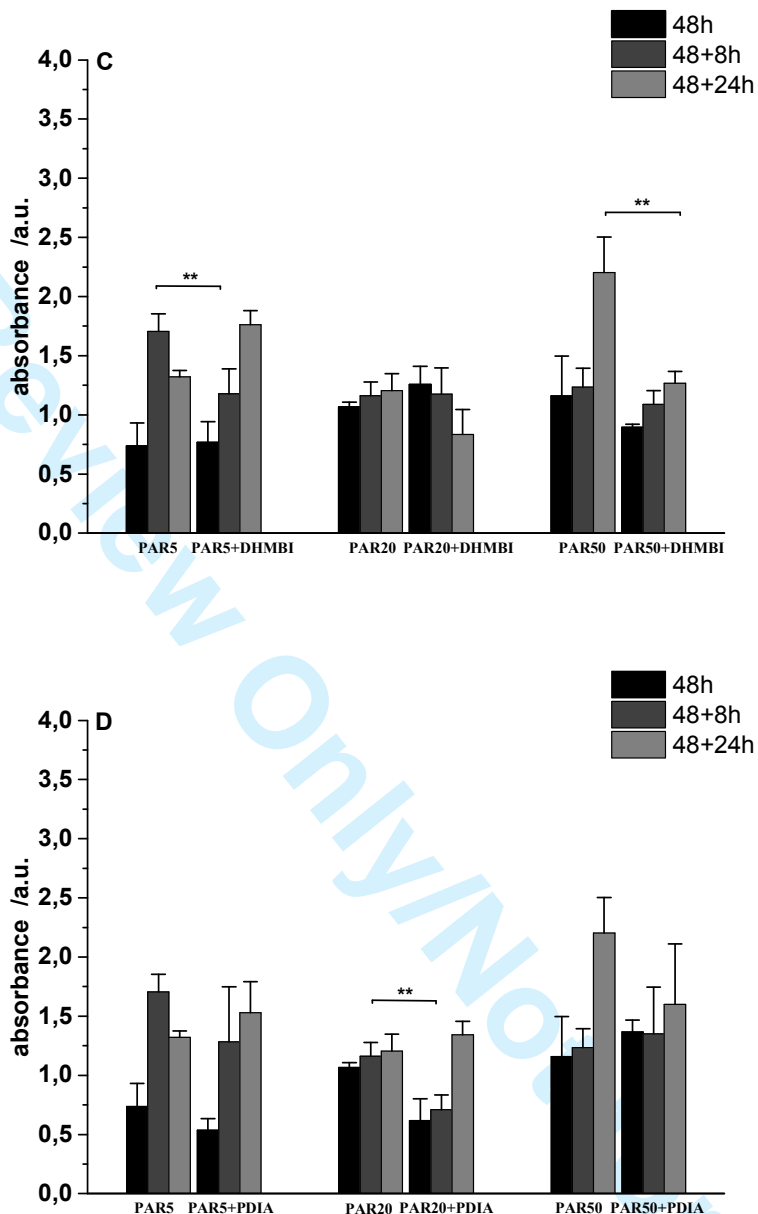
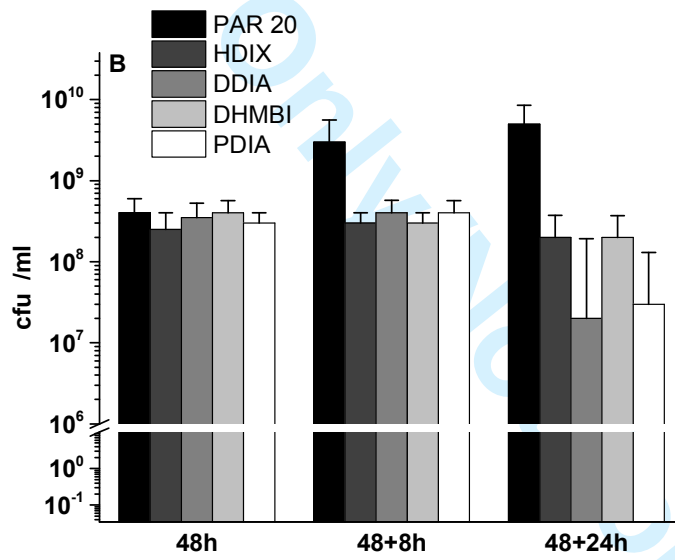
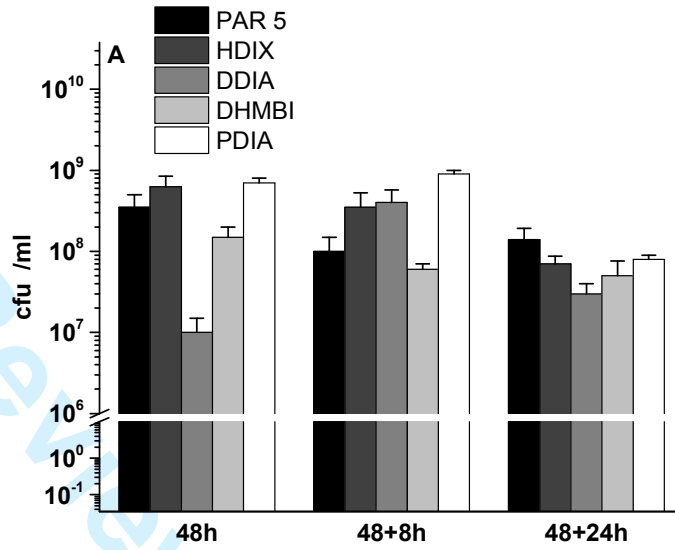


FIG. 5. Effects of the tested iminosugars compounds on mature *P. aeruginosa* biofilm (A: HDIX, B: DDIA, C: DHMBI and D: PDIA). Compounds were added to the bacteria culture 48 h after induction of biofilm. Biofilm development was monitored by absorbance after Red Congo staining at different time points. * $p < 0.05$ and ** $p < 0.01$

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3 No significant effect of the substances was observed when numbers of viable PAR
4 cells were measured in mature biofilm (Fig. 6). Although some differences were noted, for
5 example growth PAR5 and PAR20 strains was slightly inhibited (less than 1 log reduction) by
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10 some compounds, these were not significant.
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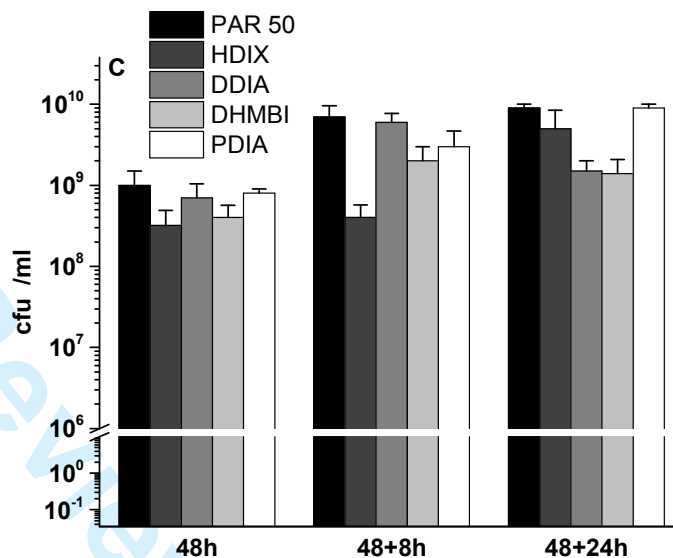


FIG. 6. Effect of the compounds on numbers of viable *P. aeruginosa* populations in 48h-old *P. aeruginosa* mature biofilm. Results depict logs of CFU/ml for three *P. aeruginosa* strains (A: PAR 5, B: PAR 20 and C: PAR 50) measured at different time intervals. There were also no significant differences between the tested strains and between the compounds and controls.

Observations on differences between activity of the tested iminosugars exerted against early biofilm formation and already formed mature biofilm of the tested strains were also supported using visualization under SEM. As shown in Fig. 7, strain PAR 5 treated with two selected compounds: HDIX and DDIA for 24 hours in the case of early biofilm and for 48+24 hours in the case of mature biofilm, for both experimental models (planktonic and 48h old forms) was taken as example to demonstrate the influence of the tested compounds on biofilm synthesis by SEM visualization.

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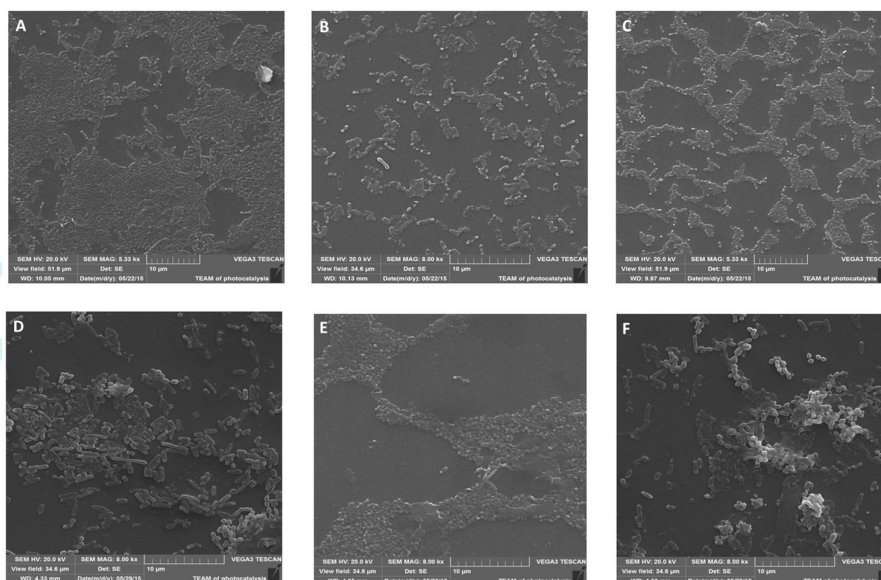


FIG. 7. SEM images representing **A: PAR 5 alone, B: PAR 5 + HDIX, C: PAR 5 + DDIA** after incubation for 24 hours (early biofilm) and **D: PAR 5 alone, E: PAR 5 + HDIX, F: PAR 5 + DDIA** after 48+24 hours incubation (48h old mature biofilm). Note differences in 3-dimensional structures in early and mature biofilms under influence of the compounds.

Incubation of PAR 5 with both selected compounds resulted in inhibition of early biofilm formation. The effect of HDIX was much stronger than that of DDIA (Fig. 7 B). No visible influence of these two compounds on 48h-old PAR 5 biofilm was seen after 24 hours incubation time (Fig. 7 D-F).

Except for investigating interactions of the iminosugar compounds with *P. aeruginosa* bacteria and biofilm formation, they were checked for their effects on human intestinal epithelium cells of the Caco2 line. A discrete necrosis but not apoptosis was observed. As can be seen in Fig. 7 cells stained entirely on the red color represent the necrotic cells after disruption of the cell membrane while the cells stained in blue represent undamaged cells.

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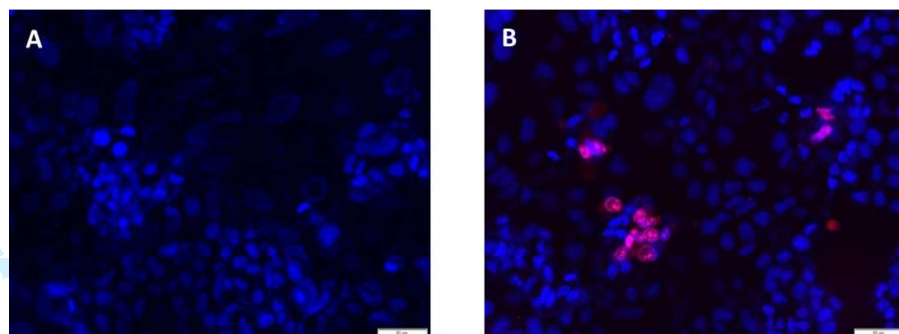


FIG. 7. **A:** Caco2 cell line cells treated with DMEM (10% FBS) – control. **B:** Caco2 cell line cells treated with **HDIX** at a concentration of 0.9 mM. Observation under a fluorescence microscope at 400^o× magnification.

The percentage of live, necrotic and apoptotic cells after 24 hours of incubation with compounds (0.9 mM) is presented in Table 1.

TABLE 1. THE EFFECT OF COMPOUNDS: **HDIX**, **DDIA**, **DHMBI** AND **PDIA** (0.9 mM) ON Caco2 CELL LINE AFTER 24 HOURS OF INCUBATIONS

Cell line Caco2	Live cells [%]	Necrotic cells [%]	Apoptotic cells [%]
DMEM (10% FBS) – negative control	99.4	0.6	0
HDIX	93,4	6.6	0
DDIA	95,4	4.6	0
DHMBI	96.7	3.3	0
PDIA	90.7	9.3	0

Discussion

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3 *Pseudomonas aeruginosa* is a well-recognized opportunistic pathogen responsible for
4 various infections, from acute pyogenic soft-tissues infections, life-threatening ventilator-
5 related pneumonias in intubated patients and in cystic fibrosis patients up to chronic wound
6 infections in diabetic patients. All these infections are related to formation of biofilm by the
7 pathogen. Since *P. aeruginosa* cells embedded in biofilm matrix are much more resistant to
8 antibiotics, disinfectants and surfactants than planktonic cells, treatment of *P. aeruginosa*
9 infections is extremely difficult and often unsuccessful.^{2,9} Therefore, many efforts have been
10 made to prevent *P. aeruginosa* biofilm formation *in vitro* and *in vivo*. Most of the studies on
11 inhibition of biofilm formation by *P. aeruginosa* concentrated on substances which might
12 interact with cell-to-cell signaling (quorum-sensing) or on gene regulation of biofilm
13 synthesizing enzymes.^{4,27}

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28 Presented approach was based chemical compounds that might act as inhibitors of
29 enzymes and/or receptors involved in the biosynthesis of EPS. Extremely important is the fact
30 that iminosugars tested here are not present in human cells, and that they may be considered
31 as carbohydrate mimics. Apparently, since iminosugars are known to interact strongly with
32 various cellular processes by inhibition of cellular glycosidases and glycosyltransferases, they
33 may also interfere with bacterial biofilm formation.²⁰ Accordingly, iminosugars may be
34 suitable compounds to interfere with the biosynthesis of EPS.

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45 Our results suggest that, indeed, it is possible to inhibit synthesis of the biofilm at
46 early stage by using new iminosugars, although there are differences in this activity among
47 four compounds studied as well as differences among individual *P. aeruginosa* in their
48 sensitivity to this activity displayed by the same compound. This sensitivity may be related to
49 the amount and chemical composition of the biofilm produced by individual strains. On the
50 other hand, the iminosugars have shown a slight influence on mature biofilm (Fig. 5), which
51 may also indicate their mechanism of action based on inhibition of exopolysaccharide

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3 backbone synthesis only in the early stages of biofilm formation. It is of interest that only one
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5 of the compounds (**HDIX**) showed their ability to promote biofilm synthesis in the late phase
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7 of the bacterial growth (Fig. 5). Since *P. aeruginosa* biofilm thickness measured at the same
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9 time was declining in the control well even without any chemical added, it may indicate that
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11 iminosugars added served as a source of nutrients for the bacteria. Alternatively, it is possible
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13 that the compound exerted its activity on 3D structure of the late biofilm in 24 hours, as
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15 shown on SEM pictures, which corresponded with increased optical density. It is also of
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17 importance that the compounds do not inhibit the growth of the bacteria forming the biofilm
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19 (Fig. 4 and 6). Our data suggest that the iminosugars interfere only with biofilm biosynthesis
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21 but not interact with reproduction cycles of the tested *P. aeruginosa* strains. Moreover, the
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23 compounds did not cause cytotoxicity towards the tested human cell line (Fig. 8 and Table 1).
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28 At the moment, it seems that synthesis and selection of the appropriate iminosugars
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30 will bring new perspectives into development of compounds, which will be able to effectively
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32 inhibit the formation of the successive stages of the exopolysaccharides chains and in
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34 consequence may increase the permeability of the biofilm to antibiotics, disinfectants or
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36 nanocompounds. These iminosugars may interfere not only with the transferases, but also
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38 with other carbohydrate-active enzymes. Undoubtedly, more studies are needed to elucidate
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40 molecular mechanism of the inhibitory activity of the iminosugars and to define specificity
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42 and anti-biofilm spectrum of the compounds against a broader range of bacterial species.
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46 In summary, it appeared that all iminosugars were able to cause decrease in
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48 *P. aeruginosa* biofilm formation in the early stage of its biosynthesis. This may indicate that
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50 synthesis of exopolysaccharides plays an important role in early biofilm formation by this
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52 pathogen. Moreover, it is plausible that inhibition of glucosyltransferases and other enzymes,
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54 which are involved in exopolysaccharides synthesis has a direct impact on *P. aeruginosa*
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56 biofilm formation and development.
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3 On the basis of the presented results, it may be speculated that showed here for the
4 first time iminosugars inhibiting early biofilm formation, will appear helpful in preventing of
5 *P. aeruginosa* biofilm formation in case of the infected tissues or on the surfaces of the
6 contaminated implants. Successful reduction or inhibiting of biofilm formation will potentiate
7 antibacterial activity of the antibiotics and other antibacterials.
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21 K/ZDS/002861 (2012). We would like to thank Marcin Oszajca for SEM visualization.
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28 Conflict of Interest

29 The authors declare no conflict of interest.
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36 References

- 37
38 1. Biela-Banas, A., E. Gallienne, F. Oulaïdi, M. Górecki, and O. R. Martin. 2013. An
39 improved methodology for the synthesis of 1-C-allyl imino-D-xylitol and -L-arabinitol
40 and their rapid functionalization. *Tetrahedron Lett.* **69**:3348-3354.
41
42
43 2. Bose, S., and A. K. Ghosh. 2011. Biofilms: a challenge to medical science. *J Clin Diagn*
44 *Res* **5**:127-130.
45
46
47 3. Byrd, M. S., I. Sadovskaya, E. Vinogradov, H. Lu, A. B. Sprinkle, S. H. Richardson, L.
48 Ma, B. Ralston, M. R. Parsek, E. M. Anderson, J. S. Lam, and D. J. Wozniak. 2009.
49 Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl
50
51
52
53
54
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56
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STRUS ET AL.

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2
3 exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in
4
5 Psl and LPS production. *Molecular Microbiology* **73**:622-638.
6
7
8 4. **Chen, M., Q. Yu, and H. Sun.** 2013. Novel Strategies for the Prevention and
9
10 Treatment of Biofilm Related Infections. *Int. J. Mol. Sci.* **14**:18488-18501.
11
12 5. **Chronowska, A., E. Gallienne, C. Nicolas, A. Kato, I. Adachi, and O. R. Martin.** 2011.
13 An expeditious synthesis of an analogue of (-)-steviamine by way of the 1,3-dipolar
14 cycloaddition of a nitrile oxide with a 1-C-allyl iminosugar. *Tetrahedron Lett.* **52**:6399-
15 6402.
16
17 6. **Compain, P., and O. R. Martin.** 2001. Carbohydrate mimetics-based
18 glycosyltransferase inhibitors. *Bioorg. Med. Chem.* **9**:3077-3092.
19
20 7. **Compain, P., and O. R. Martin.** 2007. Iminosugars: From Synthesis to Therapeutic
21 Applications. Wiley-VCH: Weinheim.
22
23 8. **Compain, P., O. R. Martin, C. Boucheron, G. Godin, L. Yu, K. Ikeda, and N. Asano.**
24 2006. Design and synthesis of highly potent and selective pharmacological
25 chaperones for the treatment of Gaucher's disease. *ChemBioChem* **7**:1356-1359.
26
27 9. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a
28 common cause of persistent infections. *Science* **284**:1318-1322.
29
30 10. **Cren, S., S. S. Gurcha, A. J. Blake, G. S. Besra, and N. R. Thomas.** 2004. Synthesis and
31 biological evaluation of new inhibitors of UDP-Galf transferase - a key enzyme in *M.*
32 *tuberculosis* cell wall biosynthesis. *Org. Biomol. Chem.* **2**:2418-2420.
33
34 11. **Fazli, M., T. Bjarnsholt, and K. Kirketerp-Møller.** 2009. Nonrandom distribution of
35 *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *J Clin*
36 *Microbiol* **47**:4084-4089.
37
38
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49
50
51
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STRUS ET AL.

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3 12. **Górska, S., W. Jachymek, J. Rybka, M. Strus, P. B. Heczko, and A. Gamian.** 2010.
4
5 Structural and immunochemical studies of neutral exopolysaccharide produced by
6
7 *Lactobacillus johnsonii* 142. *Carbohydrate Research* **345**:108-114.
8
9
10 13. **Islam, B., S. N. Khan, I. Haque, M. Alam, M. Mushfiq, and A. U. J. Khan.** 2008. Novel
11
12 anti-adherence activity of Mulberry Leaves: Inhibition of *Streptococcus mutans*
13
14 Biofilm by 1-Deoxynojirimycin isolated from *Morus alba*. *J Antimicrob Chemother*
15
16 **62**:751-757.
17
18
19 14. **Lipinski, T., C. Jones, X. Lemerclinier, A. Korzeniowska-Kowal, M. Strus, J. Rybka, A.**
20
21 **Gamian, and P. B. Heczko.** 2003. Structural analysis of the *Lactobacillus rhamnosus*
22
23 strain KL37C exopolysaccharide. *Carbohydrate Research* **338**:605-609.
24
25
26 15. **Machul, A., D. Mikołajczyk, A. Regiel-Futyra, P. B. Heczko, M. Strus, M. Arruebo, G.**
27
28 **Stochel, and A. Kyzioł.** 2015. Study on inhibitory activity of chitosan-based materials
29
30 against biofilm producing *Pseudomonas aeruginosa* strains. *Journal of Biomaterials*
31
32 *Applications*.
33
34
35 16. **Orgambide, G., H. S. Montrozier, P., J. Roussel, D. Trigalet-Demery, and A. Trigalet.**
36
37 1991. High heterogeneity of the exopolysaccharides of *Pseudomonas solanacearum*
38
39 strain GMI 1000 and the complete structure of the major polysaccharide. *The Journal*
40
41 *of Biological Chemistry* **266**:8312-8832.
42
43
44 17. **Oulaïdi, F., S. Front-Deschamps, E. Gallienne, E. Lesellier, K. Ikeda, N. Asano, P.**
45
46 **Compain, and O. R. Martin.** 2011. Second-Generation Iminoxylitol-Based
47
48 Pharmacological Chaperones for the Treatment of Gaucher Disease. *ChemMedChem*
49
50 **6**:353-361.
51
52
53
54
55
56
57
58
59
60

STRUS ET AL.

- 1
2
3 18. **Padro, M., J. A. Castillo, L. Gomez, J. Joglar, P. Clapes, and C. de Bolos.** 2010.
4
5 Cytotoxicity and enzymatic activity inhibition in cell lines treated with novel
6
7 iminosugar derivatives. *Glycoconj. J.* **27**:277-285.
- 8
9
10 19. **Paulsen, H.** 1999. Iminosugars as Glycosidase Inhibitors: Nojirimycin and Beyond.
11
12 Wiley-VCH, New York.
- 13
14 20. **Pedersen, L. L., and S. J. Turco.** 2003. Galactofuranose metabolism: a potential target
15
16 for antimicrobial chemotherapy. *Cell. Mol. Life Sci.* **60**:259-266.
- 17
18 21. **Peltier, P., R. Euzen, R. Daniellou, C. Nugier-Chauvin, and V. Ferrieres.** 2008. Recent
19
20 knowledge and innovations related to hexofuranosides: structure, synthesis and
21
22 applications. *Carbohydr. Res.* **343**:1897-1923.
- 23
24
25 22. **Reuter, M., A. Mallett, B. M. Pearson, and H. M. van Vliet.** 2010. Biofilm Formation
26
27 by *Campylobacter jejuni* Is Increased under Aerobic Conditions. *Appl. Environ.*
28
29 *Microbiol.* **76**:2122-2128.
- 30
31
32 23. **Richards, M. R., and T. L. Lowary.** 2009. Chemistry and biology of galactofuranose-
33
34 containing polysaccharides. *ChemBioChem* **10**:1920-1938.
- 35
36
37 24. **Ryder, C., M. Byrd, and D. J. Wozniak.** 2007. Role of polysacchrides in *Pseudomonas*
38
39 *aeruginosa* biofilm development. *Curr. Opin. Microbiol.* **10**:644-648.
- 40
41
42 25. **Stuetz, A. E., and T. M. Wrodnigg.** 2011. Iminosugars and glycosyl hydrolases:
43
44 Historical context, current aspects, emerging trends. *Adv. Carbohydr. Chem.*
45
46 *Biochem.* **66**:187-298.
- 47
48
49 26. **Suci, P. A., M. W. Mittelman, F. P. Yu, and G. G. Geesey.** 1994. Investigation of
50
51 ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents*
52
53 *Chemother.* **28**:2125-2133.
- 54
55
56
57
58
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STRUS ET AL.

- 1
2
3 27. **Vu, B., M. Chen, R. J. Crawford, and E. P. Ivanova.** 2009. Bacterial extracellular
4 polysaccharides involved in biofilm formation. *Molecules* **14**:2535-2554.
5
6
7
8 28. **Zhang, G.-L., C. Chen, Y. Xiong, L.-H. Zhang, J. Ye, and X.-S. Ye.** 2010. Synthesis of N-
9 substituted iminosugar derivatives and their immunosuppressive activities.
10 *Carbohydr. Res.* **345**:780-786.
11
12
13
14
15 29. **Zhang, R., W. Zhang, T. Hu, and T. Hu.** 2011. Dextran glucosidase: a potential target
16 of iminosugars in caries prevention. *Med. Hypotheses* **76**:574-575.
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Tables:

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26 TABLE 1. THE EFFECT OF TESTED COMPOUNDS: **HDIX, DDIA, DHMBI AND PDIA**
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28 (0.9 mM) ON Caco2 CELL LINE AFTER 24 HOURS OF INCUBATIONS
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Figure legends:

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37 **FIG. 1.** Chemical structures of the tested compounds: 1 – **HDIX**, 2 – **DDIA**, 3 – **DHMBI** and
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39 4 – **PDIA**.
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43 **FIG. 2.** The synthesis pathway of **PDIA (4)**, obtained by hydrogenation of compound **5**.
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47 **FIG. 3.** Effect of the compounds on PAR cultures in early phase of biofilm formation. (**A**:
48 biofilm amount produced by PAR 5, **B**: as above for PAR 20 and **C**: as above for PAR 50, **D**:
49 mean values for three tested strains). Biofilm formation expressed as absorbance at 492 nm
50 after Congo red staining at different time points. Results depict mean values \pm SD calculated
51 from three replicates ($n = 3$) $p < 0.05$.
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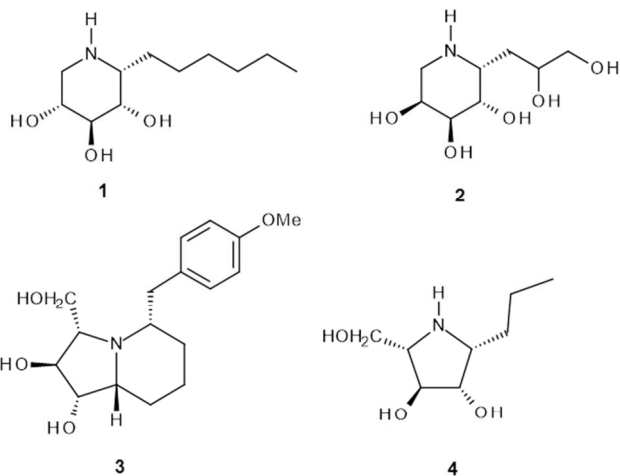
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3 **FIG. 4.** Effect of the compounds on numbers of viable *P. aeruginosa* populations in early
4 biofilm phase formation. Results depict logs of CFU/ml for three *P. aeruginosa* strains (**A:**
5 **PAR 5**, **B:** PAR 20 and **C:** PAR 50) measured at different time intervals.
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10 **FIG. 5.** Effects of the compounds on mature *P. aeruginosa* biofilm (average values for three
11 strains: PAR 5, PAR 20 and PAR 50). Compounds were added to the bacteria culture 48 h
12 after induction of biofilm. Biofilm development was monitored by absorbance after Red
13 Congo staining at different time points. $p < 0.05$.
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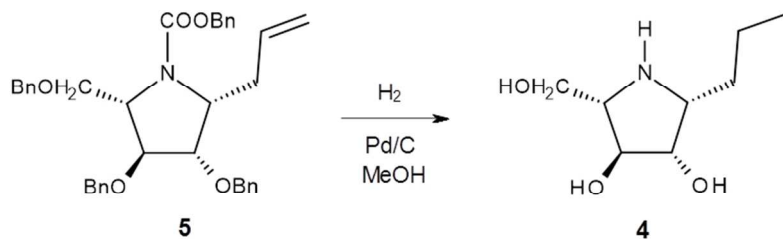
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20 **FIG. 6.** Effect of the compounds on numbers of viable *P. aeruginosa* populations in 48h-old
21 *P. aeruginosa* mature biofilm. Results depict logs of CFU/ml for three *P. aeruginosa* strains
22 (**A:** PAR 5, **B:** PAR 20 and **C:** PAR 50) measured at different time intervals.
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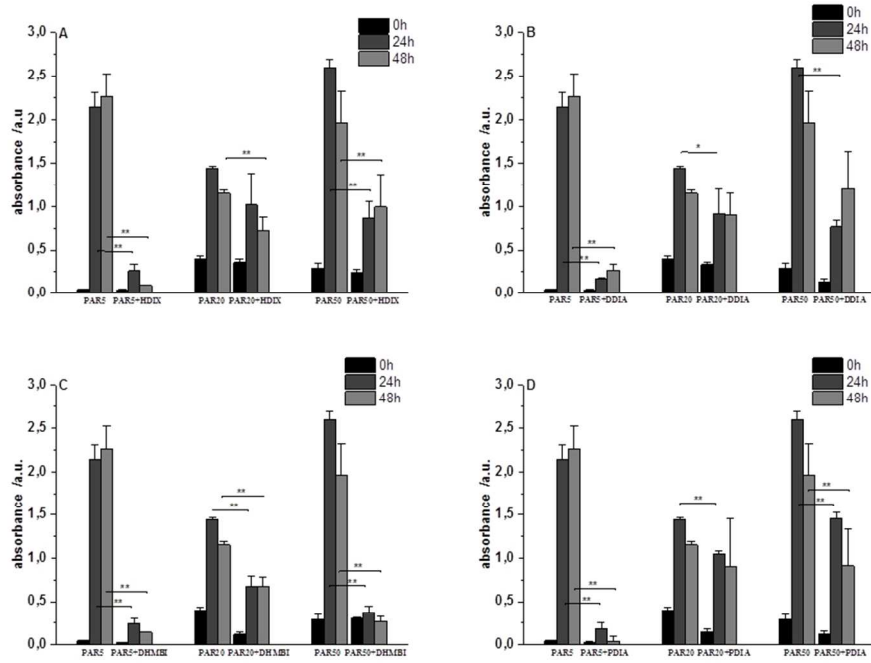
26 **FIG. 7.** SEM images representing **A:** PAR 5 alone, **B:** PAR 5 + **HDIX**, **C:** PAR 5 + **DDIA**
27 after incubation for 24 hours (early biofilm) and **D:** PAR 5 alone, **E:** PAR 5 + **HDIX**, **F:**
28 PAR 5 + **DDIA** after 48+24 hours incubation (48h-old mature biofilm).
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34 **FIG. 8. A:** Caco2 cell line cells treated with DMEM (10% FBS) – control. **B:** Caco2 cell line
35 cells treated with **HDIX** at a concentration of 0.9 mM. Observation under a fluorescence
36 microscope at 400 \times magnification.
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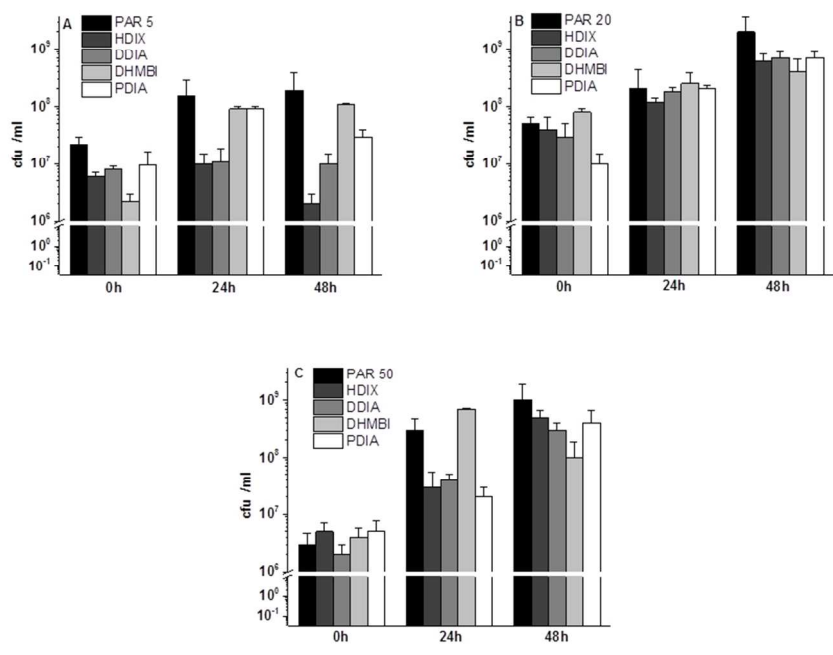
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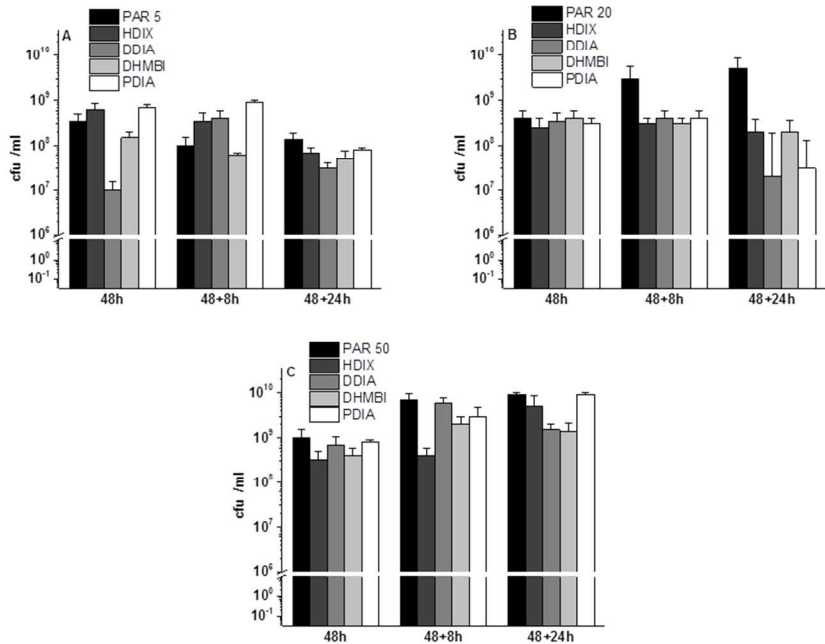
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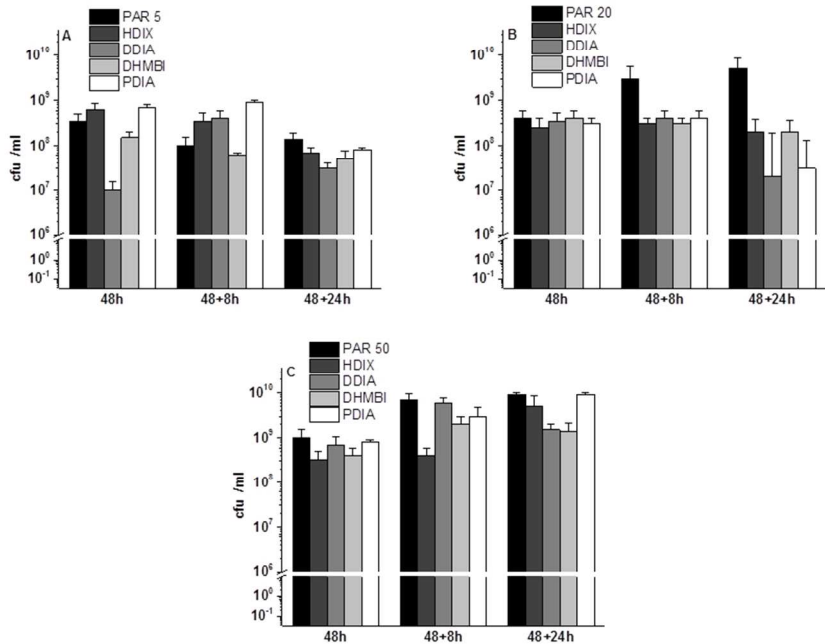
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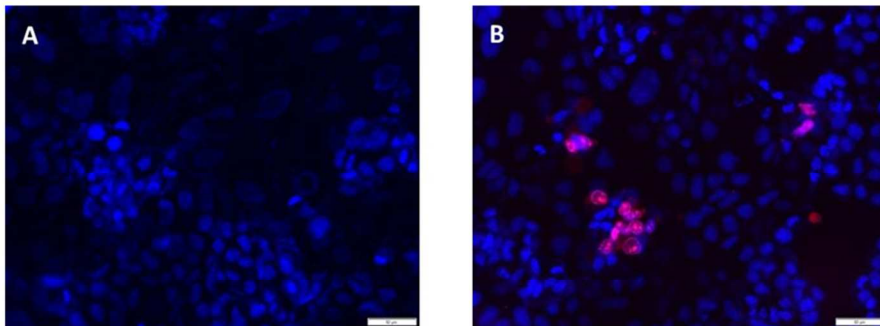
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254x190mm (96 x 96 DPI)

TABLE 1. THE EFFECT OF COMPOUNDS: **HDIX**, **DDIA**, **DHMBI** AND **PDIA** (0.9 mM)
ON Caco2 CELL LINE AFTER 24 HOURS OF INCUBATIONS

Cell line Caco2	Live cells [%]	Necrotic cells [%]	Apoptotic cells [%]
DMEM (10% FBS) – negative control	99.4	0.6	0
HDIX	93,4	6.6	0
DDIA	95,4	4.6	0
DHMBI	96.7	3.3	0
PDIA	90.7	9.3	0

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3 **FIG. 1.** Chemical structures of the compounds: 1 – **HDIX**, 2 – **DDIA**, 3 – **DHMBI** and 4 –
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5 **PDIA**.

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11 **FIG. 2.** The synthesis pathway of **PDIA (4)**, obtained by hydrogenation of compound **5**.

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15 **FIG. 3.** Effect of the tested iminosugars on PAR cultures in early phase of biofilm formation.
16 (A: **HDIX**, B: **DDIA**, C: **DHMBI** and D: **PDIA**). Biofilm formation expressed as absorbance
17 at 492 nm after Congo red staining at different time points. Results depict mean values \pm SD
18 calculated from three replicates (n = 3). * p < 0.05 and ** p < 0.01
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28 **FIG. 4.** Effect of the tested compounds on numbers of viable *P. aeruginosa* populations in
29 early biofilm phase formation. Results depict logs of CFU/ml for three *P. aeruginosa* strains
30 (A: PAR 5, B: PAR 20 and C: PAR 50) measured at different time intervals.
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38 **FIG. 5.** Effects of the tested iminosugars compounds on mature *P. aeruginosa* biofilm (A:
39 **HDIX**, B: **DDIA**, C: **DHMBI** and D: **PDIA**). Compounds were added to the bacteria culture
40 48 h after induction of biofilm. Biofilm development was monitored by absorbance after Red
41 Congo staining at different time points. ** p < 0.01
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52 **FIG. 6.** Effect of the compounds on numbers of viable *P. aeruginosa* populations in 48h-old
53 *P. aeruginosa* mature biofilm. Results depict logs of CFU/ml for three *P. aeruginosa* strains
54 (A: PAR 5, B: PAR 20 and C: PAR 50) measured at different time intervals.
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6 **FIG. 7. A:** Caco2 cell line cells treated with DMEM (10% FBS) – control. **B:** Caco2 cell line
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8 cells treated with **HDIX** at a concentration of 0.9 mM. Observation under a fluorescence
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10 microscope at 400^o× magnification.
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