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New benzyldiazepane derivatives able to reduce biofilm formation in *Escherichia coli*

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ABSTRACT

Antimicrobial resistance (AMR) represents a serious problem for human health and arises through several mechanisms. Bacteria can further enhance their intrinsic resistance by forming biofilms, defensive barriers that protect bacterial cells from antibiotics and the host immune system. Moreover, sub-inhibitory concentrations of antibiotics, such as chloramphenicol (CHL), stimulate biofilm development. A valid strategy to counteract biofilm-associated resistance is the use of biofilm formation inhibitors. For this purpose, a series of benzyldiazepane derivatives was synthesized as new anti-biofilm agents. First, their minimum inhibitory concentration (MIC) values and their effect with CHL on bacterial growth inhibition were evaluated. Then, each molecule was studied alone and in combination with sub-inhibitory concentrations of CHL to assess its ability to inhibit biofilm formation in the *Escherichia coli* K-12 strain. Four compounds showed intrinsic anti-biofilm activity and, interestingly, a combinatorial effect with CHL was observed for almost all derivatives. Overall, the results highlight benzyldiazepane as a promising scaffold for the development of anti-biofilm agents, since the tested compounds were able to decrease biofilm formation. In particular, the nitrobenzyl derivative **2** significantly reduced biofilm formation both alone and in combination with CHL.

The large use and misuse of antibiotics have led to the emergence of bacterial antimicrobial resistance (AMR), which represents a serious threat to human health. Recent studies estimate that in 2019, AMR was directly responsible for 1.27 million deaths and associated with 4.95 million deaths[1]; moreover, this number is expected to increase to 10 million by 2050.[2] AMR can appear through different mechanisms, including reduced penetration of antimicrobial agents into bacterial cells, overexpression of efflux pumps (EPs) that actively extrude antibiotics into the extracellular environment, and inactivation of antibacterial drugs or modification of their targets.[3,4] In addition, bacteria produce biofilms that further reinforce their intrinsic antibiotic resistance.[5,6]

Bacterial biofilms are microbial communities encased in a self-produced matrix, the extracellular polymeric substances (EPS), composed of polysaccharides, lipids, proteins, and extracellular DNA.[7,8] This three-dimensional matrix functions as a dual defensive barrier for microorganisms: it restricts antibiotic penetration through charge-mediated binding interactions and physically limits molecular diffusion toward embedded bacterial cells.[9] Nowadays, biofilms are recognized as an important issue in the management of human diseases due to their notorious resistance, achieving a 10- to 1000-fold higher tolerance to conventional antimicrobial agents than the corresponding planktonic bacteria.[10] Biofilm-associated resistance is multifactorial and results from the combined action of several mechanisms, including restricted antimicrobial penetration through the exopolysaccharide matrix, reduced bacterial growth rates within biofilms caused by nutrient and oxygen limitation, accumulation of metabolic wastes, Quorum-Sensing (QS) signaling, and the expression of several cluster genes encoding efflux pumps involved in antibiotic resistance.[5,11,12]

Bacterial biofilms can be organized on both abiotic surfaces, such as

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medical devices, and biotic surfaces, including human tissues, and cause almost 80% of chronic and recurrent bacterial infections.[10] Beyond serving as reservoirs of pathogenic bacteria, biofilms are also significant contributors to inflammation; during a biofilm infection, both the innate and acquired host immune systems fail to eliminate bacteria, thereby enhancing tissue damage.[13] Notably, ESKAPE pathogens frequently form biofilms, causing persistent and difficult-to-treat infections, like those affecting Cystic Fibrosis patients.[10,14] Therefore, the development of new strategies to eradicate bacterial biofilms is an urgent need.

Moreover, exposure to sub-inhibitory antibiotic concentrations (sub-MICs) is increasingly recognized as a key factor influencing bacterial physiology and pathogenesis.[15,16] Antibiotic gradients arising from clinical use, environmental release, and natural production frequently expose bacteria to sub-MIC levels, which are common during suboptimal dosing, poor tissue penetration, pharmacokinetic decline, and within biofilms. Rather than inhibiting growth, sub-MICs can promote bacterial persistence, virulence, horizontal gene transfer, and the emergence of antibiotic resistance.[15]

A prominent response to sub-MIC exposure is the induction of biofilm formation, mediated by adaptive stress pathways such as cyclic-di-GMP signaling, the stringent response, and increased extracellular matrix production. This phenomenon is widespread across bacterial species and antibiotic classes.[17,18] In *Escherichia coli*, sub-MIC concentrations of ribosome-targeting antibiotics, such as chloramphenicol, induce biofilm formation through metabolic dormancy, highlighting a stress response distinct from the oxidative stress–driven pathways associated with bactericidal antibiotics.[19,20]

Recent evidence demonstrates that efflux pumps exert direct effects on biofilm formation through multiple mechanisms[21]: *(i)* facilitation of autoinducer export required for QS-dependent biofilm development, *(ii)* enhancement of tolerance to antimicrobial compounds within biofilm structures, and *(iii)* modulation of gene expression involved in EPS production.[22] These pumps significantly contribute to biofilm-associated resistance by preventing the intracellular accumulation of antimicrobial agents that would otherwise disrupt biofilm matrix integrity and bacterial viability.[23] Moreover, several efflux pump inhibitors (EPIs), such as phenyl-arginine β-naphthylamide (PAβN), thioridazine, 1-napthylmethyl-piperazine (NMP), and quinoline derivatives, have been shown to reduce biofilm formation in both Gram-negative and Gram-positive bacteria.[24–26]

Our group previously discovered 1-benzyl-1,4-diazepane (1-BD) (Fig. 1) as an interesting EPI. 1-BD potentiated the effects of co-administered levofloxacin and enhanced ethidium bromide accumulation in *E. coli* strains overexpressing the AcrAB and AcrEF efflux pumps.[27] Preliminary studies have shown that 1-BD is capable of counteracting biofilm formation in an *E. coli* strain (unpublished data). Based on this evidence, we modified the chemical structure of 1-BD to identify new biofilm inhibitors. For this purpose, a series of benzyldiazepane derivatives carrying different electron-donating or electron-withdrawing substituents on the benzyl ring was designed and synthesized (Fig. 1). Compounds **1–9** were initially evaluated for their effects on bacterial growth by checkerboard assays in the *Escherichia coli* K-12 strain. Subsequently, their ability to inhibit biofilm formation, both alone and in combination with chloramphenicol (CHL), was evaluated in the same strain.

Compounds **1–8** were prepared following the general procedure reported in Scheme 1 (for the experimental details, see the Supplementary Material): reaction yields were reported in bracket next to the corresponding derivative. The proper benzyl halide (3-nitrobenzyl bromide, 4-nitrobenzyl bromide, 2-nitrobenzyl bromide, 3-fluorobenzyl chloride, 4-chlorobenzyl iodide, and 3-methoxybenzyl bromide) or mesylate (4-methoxybenzyl mesylate, 2-methoxybenzyl mesylate) was first reacted through nucleophilic substitution with commercially available 1-Boc-diazepane. All benzyl halides were commercially available, except for 4-chlorobenzyl iodide, which was obtained by treatment of 4-chlorobenzyl chloride with NaI, as reported in ref. 28; mesylate intermediates were prepared starting from the corresponding alcohols, following a procedure reported in literature.[29] Deprotection of intermediates **10–17** with trifluoroacetic acid (TFA) afforded the final compounds **1–8**. Compound **9** (1-(4-fluorobenzyl)-1,4-diazepane) is commercially available. Finally, all free bases **1–9** were transformed into the corresponding dihydrochloride salts, which were used for biological evaluation.

Before evaluating the ability of compounds **1–9** to inhibit biofilm formation, preliminary microbiological tests were performed. First, the minimal inhibitory concentration (MIC) was determined for CHL and the benzyldiazepane derivatives **1–9** against the *E. coli* K-12 strain. As reported in Table 1, all compounds displayed MIC values >1600 μg/mL (which is the maximum concentration tested), except for **5**, **9** (MIC = 800 μg/mL), and **2** (MIC = 1600 μg/mL). In subsequent assays, these compounds were studied at concentrations below their MIC values to rule out any direct antibacterial effect.

To evaluate a possible synergistic effect between the tested molecules and CHL on bacterial growth inhibition, checkerboard assays were performed as described in the Supplementary Material. Even at the highest concentrations of each compound (200 μg/mL), there are no significant changes (at least two-fold reduction) in the MIC of CHL; only a partial reduction in growth compared to the control was observed (Fig. S1).

The effect of compounds **1–9** on biofilm formation was then evaluated as reported in the Supplementary Material. Each molecule was first studied alone at two different sub-inhibitory concentrations (3 and 200 μg/mL) to evaluate its intrinsic ability to inhibit biofilm formation. As reported in Fig. 2, molecules **1**, **3**, **7**, and **8** did not significantly affect biofilm formation compared to the control at both concentrations. Instead, derivatives **2**, **4**, **5**, and **9** significantly ($p < 0.01$) reduced biofilm formation at the highest concentration used (200 μg/mL). In particular, reductions of 29%, 30%, 72% and 57%, respectively, were observed relative to the control. Finally, two compounds (**4** and **6**) significantly ($p < 0.01$) improved biofilm formation compared to the control when tested at 3 μg/mL.

After confirming that sub-inhibitory concentrations of CHL (0.5 and 1 μg/mL) significantly stimulated biofilm formation in *E. coli* K-12 (Fig. S2), as reported in previous studies[19,20] (see the Introduction), these two concentrations of CHL were combined with molecules **1–9** used at 3 and 200 μg/mL, as in the previous test. Two-way ANOVA analysis showed a significant interaction between CHL and all tested

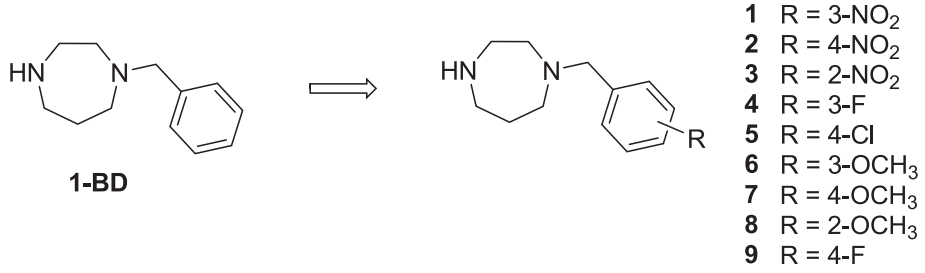
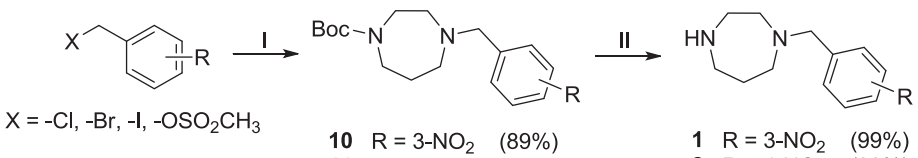


Fig. 1. Chemical structures of the benzyldiazepane derivatives **1–9** evaluated in this study.



10	R = 3-NO_2	(89%)	**1**	R = 3-NO_2	(99%)
11	R = 4-NO_2	(84%)	**2**	R = 4-NO_2	(99%)
12	R = 2-NO_2	(78%)	**3**	R = 2-NO_2	(98%)
13	R = 3-F	(78%)	**4**	R = 3-F	(89%)
14	R = 4-Cl	(58%)	**5**	R = 4-Cl	(56%)
15	R = 3-OCH_3	(83%)	**6**	R = 3-OCH_3	(73%)
16	R = 4-OCH_3	(52%)	**7**	R = 4-OCH_3	(89%)
17	R = 2-OCH_3	(45%)	**8**	R = 2-OCH_3	(70%)

Scheme 1. Reagents and conditions: I) 1-Boc-diazepane, dry Et_3N, dry CH_2Cl_2 or ethanol-free $CHCl_3$, rt., overnight (yields 45–89%); II) TFA, dry CH_2Cl_2, rt., 18 h (yields 56–99%).

Table 1
MICs of CHL and compounds **1–9** in the *E. coli* K12 strain expressed in μg/mL.

CHL	1	2	3	4	5	6	7	8	9
8–16	> 1600	1600	> 1600	> 1600	800	> 1600	> 1600	> 1600	800

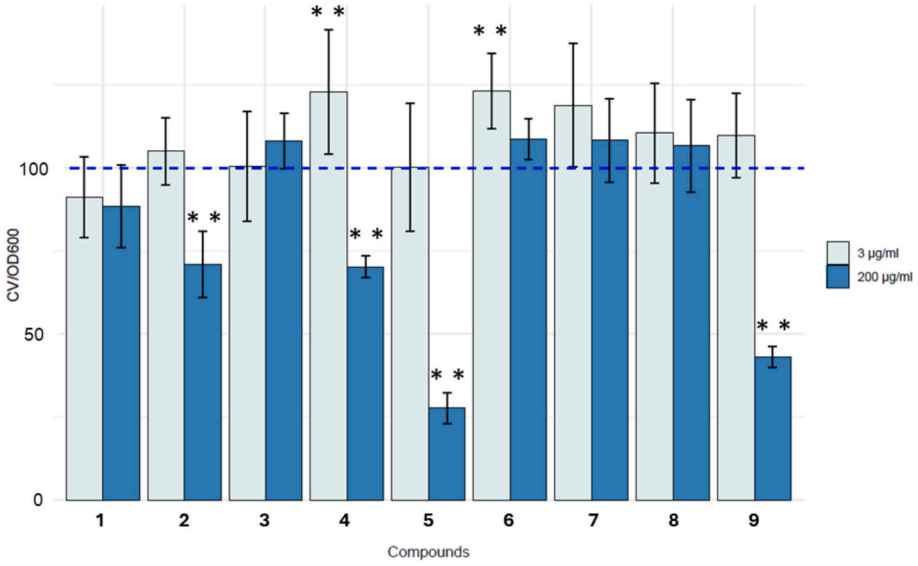


Fig. 2. Biofilm formation of the *E. coli* K-12 strain in the presence of two different concentrations (3 and 200 μg/mL) of each compound. The measurements obtained from the crystal violet test were normalized on the total growth of the strain (OD600 before the test) in each well. Data are reported as a percentage of biofilm formed in comparison with the control (growth without compounds). ** $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules, which, however, gave different results on biofilm formation depending on the tested compound and the concentration used (Fig. 3). The combination of derivatives **5** and **9**, tested at 200 μg/mL with 1 μg/mL CHL, caused a highly reduction (below 30% of the control growth) in *E. coli* growth. Since a reduction in biofilm formation under these conditions could be simply due to a decrease in the number of bacterial cells rather than to an effect of the tested molecules, data for 200 μg/mL of compounds **5** and **9** with 1 μg/mL CHL were not reported.

At the lowest concentration of 3 μg/mL, all molecules tested in combination with 0.5 μg/mL CHL stimulated biofilm formation. A similar trend was observed with 1 μg/mL CHL for most compounds, except for derivatives **5** and **9**, which displayed no significant effect, and **1**, which was able to reduce biofilm formation (Fig. 3). At the highest concentration of 200 μg/mL, nitrobenzyldiazepane derivatives **1–3** inhibited biofilm formation at both CHL concentrations. In particular, the reduction levels of 47% and 87% were observed, compared to the control, for compound **1** in combination with 0.5 and 1 μg/mL CHL, respectively; compound **2** reduced biofilm formation by 49% and 80%, while compound **3** produced reductions of 58% and 65%.

Derivatives **5** and **9**, which displayed a 4-halobenzyl moiety, caused a 72% and a 70% reduction of biofilm formation, respectively, when tested at 200 μg/mL in combination with 0.5 μg/mL CHL. As mentioned before, when **5** and **9** were studied with 1 μg/mL of the antibiotic, the effect on biofilm was not evaluated due to excessive growth inhibition. Moreover, compounds **4** and **6**, characterized by a 3-substituted benzyl ring, significantly reduced biofilm formation (64% and 55%, respectively) only in the presence of 1 μg/mL CHL. In contrast, derivatives **7** and **8**, with a 4- or 2-methoxy group on the benzyl ring, respectively, showed no effects in reducing biofilm formation (Fig. 3).

Interestingly, molecules **2**, **5**, and **9**, which were able to reduce biofilm formation on their own at 200 μg/mL, maintained strong anti-biofilm activities when used at this concentration in combination with 0.5 μg/mL CHL, and compound **2** reduced biofilm formation also with 1 μg/mL CHL.

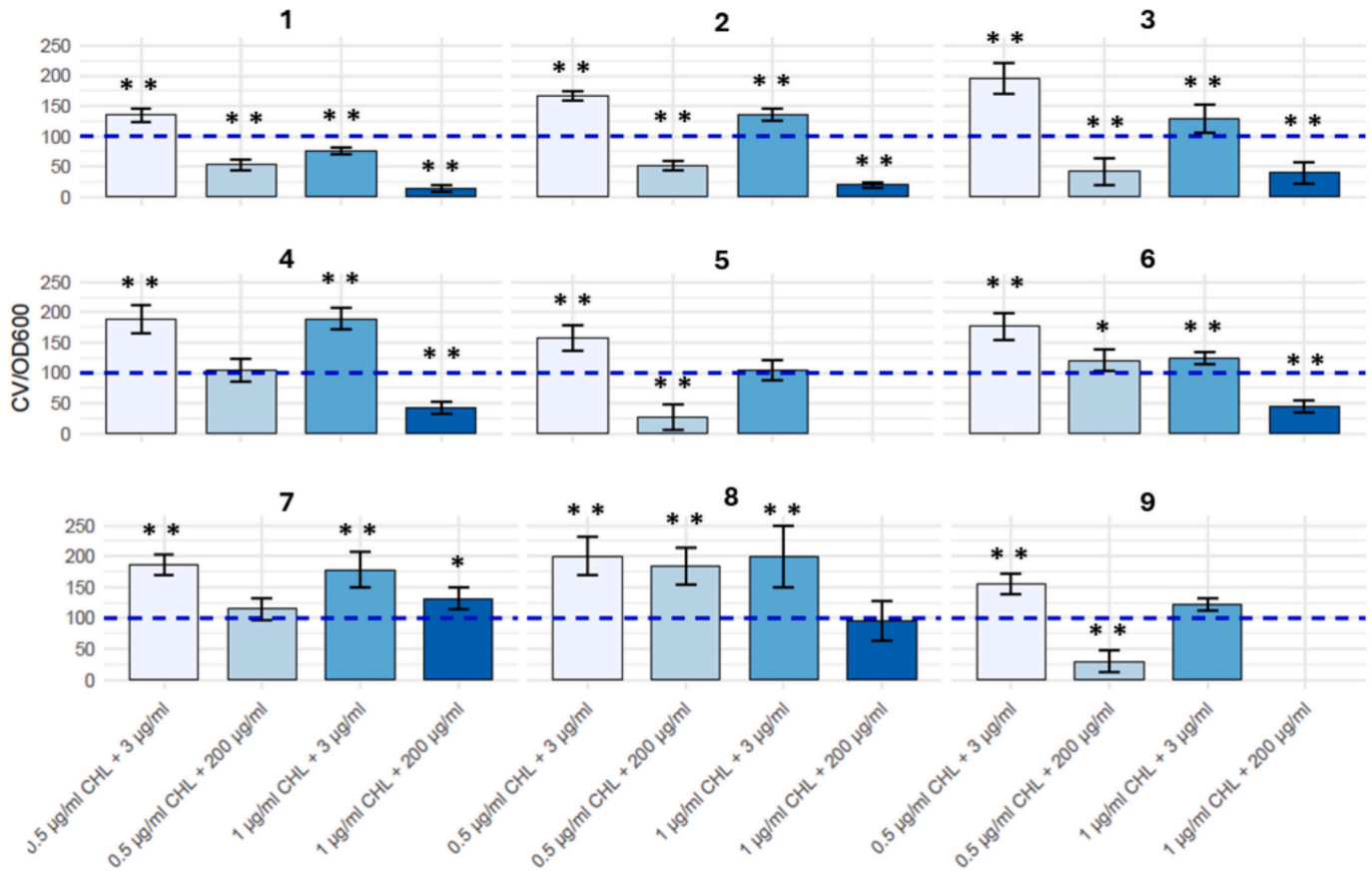


Fig. 3. Biofilm formation of *E.coli* K-12 in the presence of two sub-inhibitory concentrations of CHL (0.5 and 1 µg/mL) in combination with the two concentrations of each compound (3 and 200 µg/mL). The measurements obtained from the crystal violet test were normalized on the total growth of the strain (OD600 before the test) in each well. Data are reported as % of biofilm formed in comparison with the control (growth without compounds). *$p < 0.05$, ** $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In this work, the anti-biofilm activity of a new series of benzyldiazepane derivatives was evaluated in the *E. coli* K-12 strain. None of the compounds significantly altered the MIC value of co-administered CHL in checkerboard assays. These molecules were then tested both alone and in combination with two sub-inhibitory concentrations of CHL to verify their ability to inhibit biofilm formation in the *E. coli* K-12 strain. Only four compounds displayed intrinsic anti-biofilm effects when tested alone. However, an interesting combinatorial effect with CHL was observed, depending on the tested compound and the concentration used. Indeed, at the highest concentration tested, all compounds, except **7** and **8**, were able to significantly reduce *E. coli* biofilm formation in the presence of CHL. In general, the presence of an electron withdrawing group (nitro or halogen) is favourable for activity, thus the most promising compounds were the nitrobenzyl derivatives **1–3**, which at 200 µg/mL displayed biofilm reductions ranging from 47% to 87% at both CHL concentrations. Halogen derivatives **5** and **9** caused the greatest reduction in biofilm formation (72% and a 70%, respectively) when tested in combination with 0.5 µg/mL CHL, while **4** reduced biofilm formation only with 1 µg/mL CHL. As for the position of the substituent on the benzyl group, however, it is not easy to find a rational explanation, although the 4-position seems to confer the highest activity. Instead, the presence of the electron-donating methoxy group negatively influences the activity, as compound **6** reduced biofilm formation only in the presence of 1 µg/mL CHL, while compounds **7** e **8** showed no anti-biofilm effects.

To investigate the mechanism of action of these compounds, we evaluated their ability to inhibit bacterial efflux pumps in the *E. coli* K-12 strain. However, derivatives **1–9** at 200 µg/mL do not act as EPIs, as they do not affect the intracellular accumulation of Hoechst 33342, a known substrate of bacterial efflux pumps (Fig. S3).

In conclusion, all these data suggest that these derivatives were able to decrease biofilm formation in the *E. coli* K-12 strain. Thus, the benzyldiazepane scaffold proved to be a promising structure for the development of new anti-biofilm agents, such as compound **2**, which was able to reduce biofilm formation both alone and in combination with CHL. Further studies are needed to improve the anti-biofilm efficacy of these compounds and to elucidate their mechanism of action, such as interference with quorum sensing, or alteration of cell membrane functionality.

CRediT authorship contribution statement

Laura Braconi: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Data curation, Conceptualization. **Elena Perrin:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Andrea Carlotta Conti:** Investigation. **Giambattista Marotta:** Writing – review & editing, Investigation. **Lorenzo Mattolini:** Writing – review & editing, Investigation. **Gianluca Bartolucci:** Investigation, Data curation. **Dina Manetti:** Writing – review & editing, Validation. **Maria Novella Romanelli:** Writing – review & editing, Validation, Supervision. **Elisabetta Teodori:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Letizia Crocetti:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2026.130649.

Data availability

Data will be made available on request.

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